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Serum IL6 as a Prognostic Biomarker and IL6R as a Therapeutic Target in Biliary Tract Cancers

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Abstract

Purpose: Biliary tract cancer (BTC) is a heterogeneous group of rare gastrointestinal malignancies with dismal prognosis often associated with inflammation. We assessed the prognostic value of IL6 and YKL-40 compared with CA19–9 before and during palliative chemotherapy. We also investigated in mice whether IL6R inhibition in combination with gemcitabine could prolong chemosensitivity.

Experimental Design: A total of 452 Danish participants with advanced (locally advanced and metastatic) BTC were included from six clinical trials (February 2004 to March 2017). Serum CA19–9, IL6, and YKL-40 were measured before and during palliative treatment. Associations between candidate biomarkers and progression-free survival (PFS) and overall survival (OS) were analyzed by univariate and multivariate Cox regression. Effects of inhibiting IL6R and YKL-40 were assessed *in vitro*, and of IL6R inhibition *in vivo*.

Results: High pretreatment levels of CA19–9, IL6, and YKL-40, and increasing levels during treatment, were associated with short PFS and OS in patients with advanced BTC. IL6 provided independent prognostic information, independent of tumor location and in patients with normal serum CA19–9. ROC analyses showed that IL6 and YKL-40 were predictive of very short OS (OS < 6 months), whereas CA19–9 was best to OS > 1.5 years. Treatment with anti-IL6R and gemcitabine significantly diminished tumor growth when compared with gemcitabine monotherapy in an *in vivo* transplant model of BTC.

Conclusions: Serum IL6 and YKL-40 are potential new prognostic biomarkers in BTC. IL6 provides independent prognostic information and may be superior to CA19–9 in certain contexts. Moreover, anti-IL6R should be considered as a new treatment option to sustain gemcitabine response in patients with BTC.

Introduction

Patients with biliary tract cancer (BTC) have a dismal prognosis with a 1-year survival rate below 50% (1). This is caused by late stage at diagnosis, limited treatment options, and early emergence of treatment resistance. No diagnostic biomarkers are currently available. Serum CA19–9 is the most widely examined prognostic biomarker, but it cannot be used in 10% of the population who are Lewis antigen negative. A relevant threshold for serum CA19–9 remains unclear in BTC and has been extrapolated from patients with pancreatic cancer. However, factors such as cholestasis and smoking result in high serum CA19–9 that may not be associated with prognosis. An increased number of prognostic biomarkers with clinical utility at presentation and during treatment of BTC may enable better management and increased insight into disease nuances in the BTC population as a whole.

BTC is characterized by a dense desmoplastic tumor microenvironment (TME), in which chronic inflammation plays a cardinal role (2). Tumor-promoting inflammation in cancer is driven by interactions between cancer and stromal cells controlled by growth factors, cytokines, and signaling factors (2, 3). This interplay is essential in tumor growth and metastasis (3). Proinflammatory factors facilitate a systemic state of inflammation (4, 5) and are implicated in cancer-associated fatigue, anorexia, and cachexia, as well as reduced treatment response and poor survival (6, 7).

IL6 is a proinflammatory cytokine with a central role in sustaining chronic inflammation (8) and promotes cancer (4, 9), cachexia (10), and prometastatic niche formation in the liver (11). TME signaling stimulates IL6 production by macrophages, neutrophils, cancer-associated fibroblasts, and endothelial and cancer cells, further supporting feedback-associated TME adaptation during disease progression and treatment (12). Downstream signaling is mediated through the canonical JAK/STAT3 pathway (13) upon IL6 binding to membrane bound IL6R or through transsignaling mediated by a complex of IL6 and decoy soluble IL6R (8). The IL6/JAK/STAT3 axis is a pivotal malignant rheostat consistently activated in BTC through *SOCS3* promoter hypermethylation or aberrantly expressed miRNA. Ultimately, this activation promotes cell survival, growth, inhibition of apoptosis, release of other cytokines, and reduced treatment response (14–16).

YKL-40 [also named chitinase-3-like-1 protein (CHI3L1)] is a glycoprotein, which plays a role in inflammation, remodeling of the extracellular matrix, angiogenesis, protection against apoptosis, and metastasis (17–22). It is upregulated in many different cancers and produced by cancer, immune, and stromal cells. Expression of YKL-40 is induced by cytokines, such as IL1b, IL6, and IL13, whereas miR-24, miR-449a, and miR-342–3p can inhibit its expression (23). YKL-40 serves as a binding partner for carbohydrate residues of the glycoconjugates of the extracellular matrix and the cell, and it can also interact with receptors such as, IL13Rα2, CRTH, PAR-2, CD44, syndecan-1, and RAGE, triggering signaling cascades related to ERK, MAPK, and AKT (24–26).

Circulating levels of IL6 (25) and YKL-40 (27) are emerging as prognostic biomarkers in patients with different types of cancer, but only a small number of nonvalidated studies have examined serum IL6 and YKL-40 as prognostic biomarkers in patients with BTC (refs. 28–32; Supplementary Table S1). Both IL6 and YKL-40 have been suggested as therapeutic targets to attenuate tumor inflammation and growth (13, 18, 20, 23, 25, 33).

We tested the hypothesis that serum IL6 and YKL-40 levels before and during chemotherapy are prognostic biomarkers of overall survival (OS) in a large cohort of patients with BTC with different tumor location. In addition, we investigated the effect of IL6R and YKL-40 inhibition on BTC cellular signaling (miRNA) *in vitro*, followed by the potential clinical relevance of anti-IL6R to enhance response to gemcitabine *in vivo*.

Materials and Methods

Study design and population

We analyzed 1,590 serum samples from 452 Danish patients with advanced (locally advanced or metastatic) BTC before and during palliative chemotherapy. These patients were divided into a discovery cohort (n = 127) and a validation cohort (n = 325). The patients were included from six different clinical trials from three hospitals in Denmark between February 2004 and March 2017 (see Fig. 1; Supplementary Materials and Methods). Blood samples for biomarker analysis were drawn prior to palliative chemotherapy. Longitudinal blood samples from 311 patients were also obtained prior to the second cycle of palliative chemotherapy and at time of CT evaluation during treatment until cancer progression. Patients were followed from the time of chemotherapy initiation to time of death or last follow-up (March 6, 2018). The Danish Ethical Committee approved all protocols. Written informed consent was obtained from all included patients.

Analysis of CA19–9, IL6, YKL40, C-reactive protein, and NLR

Standard operating procedures were used for handling blood samples. Within 30 minutes to 2 hours after blood sampling, blood was centrifuged at minimum 2,300 × g for 10 minutes at 4°C, and serum was stored at −80°C until analysis. Serum IL6 and YKL-40 levels were quantified blinded to clinical data using commercial ELISA kits (Human IL-6 Quantikine HS ELISA Kit, catalog No. #HS600, R&D Systems and for YKL-40: MicroVue YKL-40 EIA, catalog No. #8040, Quidel). Serum CA19–9, C-reactive protein (CRP), neutrophil and lymphocyte counts, liver enzymes, and bilirubin were measured as part of routine care. For more information, see Supplementary Materials and Methods.

Patient serum was not available from all patients in the biobank. In the discovery cohort, serum was available for analysis of IL6 (n = 120), YKL-40 (n = 118), CA19–9 (n = 101), CRP (n = 0), and neutrophil/lymphocyte counts ratio (n = 51). In the validation cohort, serum was available for analysis of IL6 (n = 303), YKL-40 (n = 309), CA19–9 (n = 259), CRP (n = 47), and neutrophil/lymphocyte counts ratio (n = 135).

Development of a gemcitabine-resistant BTC subline *in vitro*

We developed a gemcitabine-resistant cell line (HuCCT-1res) by treating the HuCCT-1 cell line with 50 μmol/L gemcitabine for 1-hour pulse treatments across 10 consecutive cell passages (see Supplementary Materials and Methods). Gemcitabine resistance was confirmed by WST-1 Assay (Promega), as per the manufacturer's instructions. *IL6* and *IL6R* mRNA levels were measured by qRT-PCR in the gemcitabine-resistant cell line and the gemcitabine-naïve parent cell line.

In vitro treatment of human BTC cell lines with antibodies targeting IL6R and YKL-40

Five human cholangiocarcinoma cell lines (EGI-1, HuCCT-1, RBE, SNU-1079, and SSP-25) were grown in standard media and treated for 3 and 7 days with anti-IL6R (tocilizumab) and anti–YKL-40 (BIOY). The cell lines were also grown in extracellular vesicle (EV)-depleted FBS and treated with anti-IL6R (tocilizumab) or anti–YKL-40 (BIOY) for 2 and 4 days. Cell pellets and conditioned media were sent to AROS Applied Biotechnology A/S for miRNA

profiling of parent cells and EV analysis was performed using the Affymetrix GeneChip miRNA Array Platform. Data were processed using Expression Console (Affymetrix) and differentially expressed miRNAs were called using Transcriptome Analysis Control v3 (Affymetrix) software. miRNA–mRNA interactions were predicted using miRWalk 2.0 and gene set overrepresentation analysis was performed against the NetPath database. The *in vitro* miRNA alterations were compared with cholangiocarcinoma-associated miRNA changes detected in resected patient tissues from The Cancer Genome Atlas (TCGA)-CHOL miR-seq (34).

In vivo mouse studies of IL6R inhibition alone and in combination with gemcitabine

All procedures were performed in accordance with the guidelines of the Animal Care and Use Committee of the NCI, NIH (Bethesda, MD). Fourteen days after transplantation with HuCCT-1 (see Supplementary Materials and Methods), mice were randomized into four treatment groups ($n = 6$ each): (i) control, intraperitoneal injections of vehicle (PBS with 10% DMSO); (ii) gemcitabine, 15 mg/kg twice weekly; (iii) tocilizumab, 20 mg/kg weekly; and (iv) tocilizumab and gemcitabine, 20 and 15 mg/kg twice weekly, respectively. Half of the mice in group 2 (gemcitabine) were euthanized at the time when tumors in the controls (group 1) reached a combined size of 2 cm in diameter (humane endpoint). Group 3 mice (tocilizumab) were all euthanized at the humane endpoint. The remaining mice in group 2 (gemcitabine) and all mice in group 4 (gemcitabine + tocilizumab) were euthanized together when tumors in the gemcitabine group reached 2 cm in diameter. Upon mouse euthanization, serum samples were collected and tumors were weighed. Either fresh frozen (−80°C) or formalin-fixed, paraffin-embedded tissues were prepared and stored.

Statistical analyses

The biomarker study was conducted and presented according to the most recent reporting recommendations for tumor marker (REMARK) prognostic studies (35). Associations of progression-free survival (PFS) and OS with serum CA19–9, IL6, YKL-40, CRP, and neutrophil/lymphocyte ratio (NLR) levels were assessed by Kaplan–Meier plots (log-rank test) using tertiles or novel thresholds. The associations were further tested by means of Cox proportional hazards regression models on: tertiles, log-transformed continuous values, and longitudinal log-changes during treatment. The results from all analyses were subsequently presented as HRs with corresponding 95% confidence intervals (CIs). Moreover, we performed analyses adjusted for all significant baseline patient characteristics. The calculations regarding serum IL6, YKL-40, and CA19–9 were prespecified before the start of this biomarker study. The calculations regarding serum CRP, NLR, bilirubin, and liver enzymes were not prespecified. To evaluate the biomarkers ability to predict early death (<180 days), analysis by ROC with corresponding AUC metric was performed. For longitudinal analyses of dynamics during chemotherapy, biomarkers were included in the models for both PFS and OS as time-depending covariates (i.e., at any given timepoint, the most recent value at that timepoint was used). In these models, HRs were assumed to be time invariant and reported for a one unit increase on a $\log_2$ scale, while biomarker levels changed over time.

Analyses were performed in R version 3.2.2 with $P < 0.05$ considered as significant (www.r-project.org). GraphPad Prism (v7.0 and v8.0) was used for all *in vitro* and *in vivo* animal studies. Human tissue samples were analyzed by Mann–Whitney *U* test after verifying nonnormal distribution by D'Agostino–Pearson normality test. Unpaired Student *t* test was applied to calculate expression differences in qRT-PCR and *in vivo* mice studies.

Results

Pretreatment serum IL6 and YKL-40 and association with clinicopathologic features in patients with advanced BTC

Pretreatment clinical characteristics of the 452 patients with locally advanced or metastatic BTC are shown in Table 1. The patients in the discovery and validation cohorts were comparable according to age, sex, number of patients with elevated CA19–9, as well as median levels of CA19–9, IL6, and YKL-40. Compared with the discovery cohort, the validation cohort included more patients with metastatic disease, performance status (PS) ≥ 1, and elevated IL6.

There was no relationship between either IL6 or CA19–9 and anatomic location of BTC, treatment type, and presence of metastases. Higher IL6 was associated with sex in the discovery and validation cohorts, and with PS in the validation cohort only. Higher YKL-40 was associated with higher PS in both cohorts and with intrahepatic or perihilar location in the discovery cohort (Supplementary Table S2).

CA19–9 and IL6 were significantly correlated in the validation cohort only (*rho* = 0.18; $P = 0.01$). No correlation between CA19–9 and YKL-40 was found (discovery cohort: *rho* = 0.08 and validation cohort: *rho* = 0.07). IL6 correlated positively with YKL-40 (discovery cohort: *rho* = 0.47; $P < 0.01$ and validation cohort: *rho* = 0.44; $P < 0.01$).

Pretreatment serum IL6, YKL-40, and CA19–9 and PFS in patients with advanced BTC

Because there are no reference thresholds for the prognostic utility of IL6 and YKL-40 in patients with BTC, we stratified the patients into tertiles for each biomarker and tested their association with PFS using log-rank statistics and univariate Cox analysis. Patients with IL6 in the highest tertile had shorter PFS compared with patients with IL6 in the lowest tertile in both the discovery (HR, 1.80; 95% CI, 1.15–2.84) and validation (HR, 1.96; 95% CI, 1.47–2.61) cohorts. Similarly, patients with YKL-40 in the highest tertile had shortest PFS (discovery cohort: HR, 1.77; 95% CI, 1.12–2.79 and validation cohort: HR, 1.99; 95% CI, 1.50–2.65). For CA19–9, this was only found for the validation cohort (HR, 1.66; 95% CI, 1.21–2.27). In multivariate Cox analysis including tertiles of biomarkers and significant baseline characteristics (discovery cohort: metastases and validation cohort: metastases, PS, and treatment), serum IL6 (HR, 1.60; 95% CI, 1.10–2.34) and CA19–9 (HR, 1.77, 95% CI, 1.23–2.54) in the highest tertile were independently associated with shorter PFS in the validation cohort when compared with levels in the lowest tertile (Supplementary Table S3).

Supplementary Fig. S1 illustrates the Kaplan–Meier survival curves for PFS according to biomarker tertiles in patients with advanced BTC. Patients with two or three biomarkers in the highest tertiles had very short PFS in both the discovery and the validation cohorts.

As the range of serum marker tertiles varied between the discovery and validation cohorts, we also performed continuous modeling with log_2-transformed values and Cox statistics. Increasing CA19–9 was associated with shorter PFS in both the discovery (HR, 1.07; 95% CI, 1.01–1.12) and validation (HR, 1.08; 95% CI, 1.04–1.13) cohorts. Increasing IL6 (HR, 1.27; 95% CI, 1.17–1.38) trended toward significant association with shorter PFS in discovery cohort (HR, 1.13; 95% CI, 0.99–1.29) and was significantly associated with shorter PFS in the validation cohort (HR, 1.27; 95% CI, 1.17–1.38). YKL-40 was only significantly associated with shorter PFS in the validation cohort (HR, 1.25; 95% CI, 1.15–1.36). When combining continuous measurements of all three biomarkers in a multivariate Cox regression model with significant baseline characteristics (discovery cohort: metastases and validation cohort: metastases, PS, and treatment), IL6 (HR, 1.19; 95% CI, 1.07–1.32) and CA19–9 (HR, 1.07; 95% CI, 1.03–1.12) were independently associated with PFS in the validation cohort (Supplementary Table S3).

Pretreatment serum IL6, YKL-40, and CA19–9 and OS in patients with advanced BTC

Similar to PFS, there are no reference thresholds for the prognostic utility of IL6 and YKL-40 in patients with BTC. Accordingly, we stratified the patients into tertiles for each serum biomarker and tested their association with OS using log-rank statistics and univariate Cox analysis.

Patients with IL6 in the highest tertile had significantly shorter OS compared with patients in the lowest IL6 tertile in both discovery (HR, 1.94; 95% CI, 1.24–3.04) and validation (HR, 2.63; 95% CI, 1.89–3.37) cohorts. Similarly, patients with YKL-40 in the highest tertile had the shortest OS (discovery cohort: HR, 1.84; 95% CI, 1.16–2.92 and validation cohort: HR, 1.93; 95% CI, 1.46–2.55). This was also found for CA19–9 (discovery cohort: HR, 1.75; 95% CI, 1.07–2.85 and validation cohort: HR, 1.86; 95% CI, 1.35–2.55). In multivariate Cox analysis including tertiles of the biomarkers and significant baseline characteristics [discovery cohort: metastases and validation cohort: PS (0 vs. 1+2)], IL6 (HR, 2.05; 95% CI, 1.44–2.91), YKL-40 (HR, 1.41; 95% CI, 1.01–1.98), and CA19–9 (HR, 1.88; 95% CI, 1.32–2.68) in the highest tertiles were independently associated with shorter OS in the validation cohort when compared with levels in the lowest tertile, whereas the same was seen only for YKL-40 (HR, 1.90; 95% CI, 1.02–3.55) in the discovery cohort (Table 2).

Figure 2 illustrates the Kaplan–Meier survival curves for OS according to biomarker tertiles in patients with advanced BTC. Patients with two or three biomarkers in the highest tertiles had very short OS in both the discovery and the validation cohorts (Fig. 2D).

As the range of the serum biomarker tertiles varied between the discovery and validation cohorts, we also performed continuous modeling with log_2-transformed values and Cox statistics. Increasing CA19–9 was associated with shorter OS in both the discovery (HR, 1.06; 95% CI, 1.01–1.12) and validation (HR, 1.08; 95% CI, 1.04–1.13) cohorts. Increasing IL6 (HR, 1.36, 95% CI, 1.26–1.48) and YKL-40 (HR, 1.25; 95% CI, 1.15–1.36) were only significantly associated with shorter OS in the validation cohort. When combining continuous measurements of all three serum biomarkers in a multivariate Cox regression model including significant baseline characteristics (metastases in discovery cohort and PS 0 vs. 1+2 in validation cohort), IL6 (HR, 1.25; 95% CI, 1.12–1.38) and CA19–9 (HR, 1.06;

95% CI, 1.02–1.11) were independently associated with OS in the validation cohort and only CA19–9 (HR, 1.06; 95% CI, 1.00–1.11) in the discovery cohort (Table 2).

We also performed ROC curve analysis to describe the ability of IL6, YKL-40, and CA19–9 to predict death at 1 year. While the ROC curves were similar in the discovery cohort (AUC: IL6, 0.59; YKL-40, 0.58; and CA19–9, 0.59; Fig. 3A), AUC values were substantially higher for IL6 (AUC, 0.69) and YKL-40 (AUC, 0.67) than CA19–9 (AUC, 0.55) in the validation cohort (Fig. 3B). A *post hoc* analysis of the ability of IL6, YKL-40, and CA19–9 to predict survival from time of inclusion to all timepoints until 3 years (Fig. 3C) suggests that pretreatment CA19–9 was best to predict long survival (>1.5 years), whereas pretreatment IL-6 and YKL-40 were better to predict short survival (<1.5 years). This suggests that inflammation at time of diagnosis of advanced BTC is associated with rapid deterioration and that high IL6 and YKL-40 could potentially be used to identify patients with a very poor prognosis. Accordingly, we identified an IL6 threshold of 21.6 pg/mL that could predict OS < 6 months with a specificity of 0.85 and an YKL-40 threshold of 395 ng/mL that could predict OS < 6 months with a specificity of 0.85 in our cohort of patients with BTC. When applying these novel BTC-specific IL6 and YKL-40 thresholds, patients with advanced BTC and elevated IL6 (>21.6 pg/mL) or YKL-40 (>395 ng/mL) had significantly shorter OS than patients with IL6 and YKL40 below these cutoffs (Fig. 3D and E).

Pretreatment serum IL6, YKL-40, and CA19–9 and OS in patients with advanced BTC stratified by tumor location

Because BTC is a heterogeneous group of cancers, we studied the prognostic performance of these serum biomarkers according to anatomic subtypes. Because of the low number of patients in each subgroup, we combined the discovery and validation cohorts for this analysis. Univariate Cox analysis of the highest versus the lowest tertile of the biomarkers in patients stratified by tumor location showed that high IL6 was the only marker associated with short OS across all anatomic subtypes. High YKL-40 was associated with short OS in patients with intra- and extrahepatic cholangiocarcinoma, whereas high CA19–9 was associated with short OS in patients with intrahepatic cholangiocarcinoma and in gallbladder cancer (Supplementary Table S4).

Pretreatment serum IL6 and YKL-40 and OS in patients with advanced BTC and normal CA19–9

Approximately 10% of the population are Lewis antigen negative and cannot produce CA19–9. Conversely, others have normal levels despite of having been diagnosed with BTC. In our cohort of patients with advanced BTC with CA19–9 measurements, 27% ($n = 79$) had normal CA19–9 levels. In this group of patients, we found that patients with serum IL6 in the highest tertile ($n = 22$; HR, 2.40; 95% CI, 1.34–4.33) had shorter OS compared with the lowest tertile. This was not found for serum YKL-40 in the highest tertile ($n = 25$; HR, 1.58; 95% CI, 0.90–2.77).

Pretreatment serum IL6, YKL-40, and CA19–9 and OS compared with CRP and NLR in patients with advanced BTC

CRP correlated with IL6 (*rho* = 0.60; $P < 0.01$) and YKL-40 (*rho* = 0.61; $P < 0.01$; Supplementary Fig. S2). Because IL6 and YKL-40 are associated with inflammation, it should be considered whether standard inflammatory biomarkers could be as informative. When CRP (only available in the validation cohort) and NLR were included in univariate Cox analyses, we found that patients with CRP (HR, 3.97; 95% CI, 1.50–10.53) and NLR (HR, 2.34; 95% CI, 1.38–3.95) in the top tertiles had significantly shorter OS when compared with the lowest tertile in the validation cohort (Supplementary Table S5). In multivariate Cox analysis [including CRP, NLR, metastases (yes/no), and PS (≥1 vs. 0)], we found that CRP and NLR were not independently predictive of OS.

To compare the prognostic associations between these four inflammatory biomarkers and CA19–9 (highest tertiles vs. lowest tertiles), we performed univariate and multivariate Cox analysis in the 452 patients (pooled cohort) with all biomarkers determined. In univariate analysis, high IL6 (HR, 2.40; 95% CI, 1.88–3.06), YKL-40 (HR, 1.90; 95% CI, 1.50–2.42), CA19–9 (HR, 1.79; 95% CI, 1.37–2.33), NLR (HR, 1.92; 95% CI, 1.26–2.92), CRP (HR, 3.97; 95% CI, 1.50–10.53), metastatic disease, and PS 1 or 2 (vs. 0) were all significantly associated with short OS (Supplementary Table S6). In subsequent multivariate Cox analysis, IL6 was the only biomarker found to be independently associated with OS (HR, 17.98; 95% CI, 1.18–273.45).

Associations of pretreatment serum IL6 and YKL-40 with hepatobiliary function in advanced BTC

Circulating IL6 and YKL-40 levels can reflect both the tumor-driven inflammation and the systemic response to hosting a tumor (s). We evaluated the association between IL6 and YKL-40 and biomarkers of cholestasis (ALP and bilirubin), hepatic function [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)], and general tissue damage [lactate dehydrogenase (LDH)] in the combined cohort. IL6 positively correlated with ALP (*rho* = 0.43; $P < 0.01$), bilirubin (*rho* = 0.21; $P = 0.02$), AST (*rho* = 0.28; $P < 0.01$), and LDH (*rho* = 0.21; $P = 0.01$). YKL-40 only negatively correlated with ALT (*rho* = −0.18; $P = 0.04$).

Longitudinal serum IL6, YKL-40, and CA19–9 monitoring during treatment for prediction of PFS and OS

To assess the clinical utility of IL6, YKL-40, and CA19–9 for monitoring patients with advanced BTC during treatment, serial follow-up serum samples were collected. A total of 1,449 samples from 311 patients with advanced BTC were analyzed (median, 4.6 samples/patient). We investigated the association between PFS and longitudinal changes in CA19–9, IL6, and YKL-40 by calculating the HR per $\log_2$ increase from levels at inclusion. Longitudinal increases in IL6 (discovery cohort: HR, 1.33; 95% CI, 1.14–1.54 and validation cohort: HR, 1.29; 95% CI, 1.14–1.47) and YKL-40 (discovery cohort: HR, 1.19; 95% CI, 1.02–1.39 and validation cohort: HR, 1.25; 95% CI, 1.09–1.43) levels during treatment were associated with shorter PFS in both cohorts. CA19–9 level increases were associated with shorter PFS only in the discovery cohort (HR, 1.14; 95% CI, 1.02–

1.27). In multivariate Cox analysis (including all univariate significant clinical baseline characteristics), longitudinal increases in IL6 level trended toward independent association (HR, 1.30; 95% CI, 0.96–1.77) and were independently associated (HR, 1.19; 95% CI, 1.01–1.14) with PFS in the discovery and validation cohort, respectively (Table 3).

Next, we examined the association between OS and longitudinal biomarker changes. Univariate analysis in both cohorts showed that increases in IL6 (discovery cohort: HR, 1.38; 95% CI, 1.20–1.60 and validation cohort: HR, 1.32; 95% CI, 1.21–1.58), YKL-40 (discovery cohort: HR, 1.25; 95% CI, 1.07–1.47 and validation cohort: HR, 1.30; 95% CI, 1.13–1.49), and CA19–9 (discovery cohort: HR, 1.23; 95% CI, 1.11–1.36 and validation cohort: HR, 1.07; 95% CI, 1.02–1.13) during treatment were associated with shorter OS (Table 3). In multivariate Cox analysis (including the three biomarkers) and significant baseline characteristics [discovery cohort: metastasis and validation cohort: PS (0 vs. 1 or 2)], only IL6 was independently associated with OS in both cohorts (discovery cohort: HR, 1.41; 95% CI, 1.03–1.93 and validation cohort: HR, 1.25; 95% CI, 1.05–1.49). CA19–9 was independently associated with OS only in the discovery cohort (HR, 1.16; 95% CI, 1.02–1.31) and YKL-40 was not an independent biomarker (Table 3).

Finally, to elucidate the prognostic value of the individual markers, we performed a multivariate analysis of each marker, excluding the other markers, but including all univariately significant clinical baseline characteristics [PS (0 vs. 1 or 2) and metastases (no vs. yes)]. We found that changes in IL6, YKL-40, and CA19–9 during palliative chemotherapy of patients with advanced BTC were all independently associated with OS, suggesting a partial overlap of their prognostic capacity (Supplementary Table S7).

IL6 receptor as a potential therapeutic target

The significant association between temporal IL6 changes and the risk of progression and death could reflect response to chemotherapy by the tumor and/or by the host in general. To evaluate a relationship between tumor epithelial IL6 expression and gemcitabine treatment, we established a gemcitabine-resistant BTC model (HuCCT-1res) as a mimic of the putative clinical resistance phenotype. This was achieved by exposing HuCCT-1 to 50 μmol/L gemcitabine 1-hour pulse treatments over 10 passages compared with HuCCT-1 exposed once to 125 nmol/L gemcitabine for 72 hours (HuCCT-1^{sen}; Supplementary Fig. S3A). In this system, *IL6* mRNA was significantly upregulated in HuCCT-1res compared with control parental cells ($P = 0.02$), but not in HuCCT-1^{sen} (Supplementary Fig. S3B). Conversely, *IL6R* mRNA was upregulated in HuCCT-1^{sen} ($P = 0.01$), but not in HuCCT-1res (Supplementary Fig. S3C), suggesting that IL6R inhibition may counteract gemcitabine-induced epithelial IL6 upregulation.

In vitro studies of BTC cells treated with anti-IL6R and anti–YKL-40 and effects on miRNA

We evaluated the effects of anti-IL6R and anti–YKL-40 by assessing parental miRNAs and miRNA load in EVs to emphasize global network effects. Tocilizumab caused a more pronounced effect on miRNA expression in both parental cells and EVs compared with anti–YKL-40 (Supplementary Table S8). An overlap in deregulated miRNAs was found between treatment groups (Supplementary Fig. S3D). Among these differentially

expressed miRNAs, 11 anti-IL6R–associated and three anti–YKL-40–associated miRNAs were differentially expressed in BTC patient tissues, compared with surrounding normal tissues in TCGA-CHOL cohort, in the opposite direction to treatment-associated changes, suggesting potential rescue of these miRNAs with these biologics (Supplementary Table S9; Supplementary Results). Analysis of miRNA–mRNA networks suggested that these effects were related to specific signaling networks, including IL6, TGFα, and TGFβ pathways (Supplementary Fig. S4). These data indicate that IL6R and YKL-40 inhibition induced distinct intra- and intercellular responses within cardinal cytokine signaling networks in BTC cells.

Therapeutic potential of IL6R inhibition *in vivo* with and without gemcitabine

We assessed the effect of anti-IL6R treatment using tocilizumab alone or in combination with gemcitabine in mice. Athymic (nu/nu) female mice were subcutaneously transplanted with the BTC cell line, HuCCT-1, and after 14 days randomized into four treatment groups ($n = 6$ in each group) that received intraperitoneal injection of either 10% DMSO in PBS (controls), tocilizumab, gemcitabine, or gemcitabine + tocilizumab (Fig. 4A). All treatments were well-tolerated with no adverse effects, such as differences in body weight, observed hepatotoxicity, or behavioral changes (lethargic nature). Mice treated with tocilizumab monotherapy reached the endpoint (total tumor size of 2 cm in diameter) 10 days after the control mice, whereas the gemcitabine treatment arm reached the endpoint 35 days after the controls, at which point both gemcitabine and gemcitabine + tocilizumab groups were sacrificed (Fig. 4B and C). We observed that the combination of gemcitabine + tocilizumab significantly reduced tumor burden compared with gemcitabine monotherapy ($P = 0.04$; Fig. 4D).

Next, we compared the serum IL6 in these mice at time of sacrifice. Mice treated with gemcitabine had higher IL6 ($P < 0.01$) than controls (Fig. 4E). As expected, higher IL6 was found in the tocilizumab-treated groups, suggesting a positive feedback because of impaired signaling. Serum IL6 correlated with tumor weight in all mice ($P < 0.01$; $R^2 = 0.175$; Fig. 4F). No correlation was found between serum IL6 and *IL6* mRNA expression in tumor tissues. Histopathologic evaluation showed decreased mitotic activity, greater necrosis, and increased fibrosis in mice treated with gemcitabine alone and gemcitabine + tocilizumab when compared with controls (Supplementary Fig. S5A–S5D). IL6 may not be a traditional driver; however, our data suggest that it is implicated in the degree of response to gemcitabine and that inhibition of the receptor may prolong the duration of therapeutic benefit.

Discussion

This biomarker REMARK-guided multicenter study included 452 patients with advanced (locally advanced and metastatic) BTC. In both the discovery and validation cohorts, we found that high serum concentrations of CA19–9 and the inflammatory proteins, IL6 and YKL-40, prior to and during treatment were associated with poor prognosis. Interestingly, IL6 displayed significant prognostic potential in patients with normal CA19–9 and across all

tumor locations. In patients with early mortality (OS < 6 months), IL6 and YKL-40 were more prognostic than CA19–9.

As therapeutic management becomes increasingly individualized for patients with BTC, context- and patient-specific application of candidate biomarkers will also likely become important. Clinical validity is central in the evaluation of novel biomarkers, which should reproducibly demonstrate robustness across cohorts (36). This is especially important in highly heterogeneous and rare malignancies such as BTC. Consequently, heterogeneous patient cohorts are found in previously reported studies of patients with BTC with similar characteristics as our study (1, 32, 37, 38). In accordance with previous studies, metastatic disease was associated with poor prognosis, as was poor PS in the validation cohort (37, 38). The lack of association between PS and OS in the discovery cohort could be due to the lower number of patients. BTC is clinically treated homogenously despite having a heterogeneous underlying biology (1, 39). Our data suggest that serum IL6, YKL-40, and CA19–9 are not associated with specific clinical baseline characteristics and that the prognostic value of these biomarkers, most notably IL6, is not dependent on anatomic location.

Serum CA19–9 is the most evaluated prognostic biomarker in BTC and our data support the prognostic association, especially as a continuous variable prior to treatment. However, serum IL6 has previously been proposed to be associated with tumor burden and DFS (30, 40, 41), and IL6 and YKL-40 have been associated with OS in small studies of BTC (28, 29, 31), as well as in the larger ABC-03 trial for IL6 (32). Our results indicate a possible clinical utility of these inflammatory biomarkers measured at inclusion either in combination with CA19–9 or, in the case of IL6, providing warranted prognostic information in patients with BTC with normal levels of CA19–9. Implementation of serum IL6 in daily clinical practice would require a threshold that is above what is defined on the basis of a healthy population (42, 43). It remains elusive what the biological rationale is for a higher clinical threshold in BTC. However, subclinical biliary tract inflammation not directly related to prognosis may be a contributing factor (44). As such, our results confirm earlier prognostic associations of pretreatment CA19–9, as well as provide rationale for the additional inclusion of IL6 (and, to a lesser extent, YKL-40).

Inflammation is suggested as a general marker of prognosis. The best inflammatory markers to use remain undetermined, although several are suggested, for example, NLR, CRP, platelet lymphocyte ratio, or modified Glasgow prognostic score, which are often presented without including CA19–9 as comparison (37, 45, 46). Our retrospective *post hoc* analysis corroborates previous findings, suggesting a prognostic value of NLR, although inferior to serum IL6. Thus, comprehensive prospective studies are needed to elucidate the role of substitute inflammatory markers.

Intriguingly, in our *post hoc* analysis, CA19–9 at inclusion is suggested to be superior in predicting survival past 1.5 years, whereas IL6 and YKL-40 may be useful to identify patients with early mortality (short OS). This could have valuable clinical impact in selecting patients for whom a less toxic treatment or experimental protocols should be considered.

Assessment of tumor progression in patients with BTC is challenging due to diffuse and infiltrating tumors. Kinetic changes in biomarkers with prognostic value may provide useful clinical guidance when monitoring patients with BTC during treatment. Previous studies investigating biomarker kinetics in patients with advanced BTC found an association between improved prognosis and early decreases in serum CA19–9 during treatment (47, 48). Our data support these findings. Interestingly, temporal changes in IL6 may have a stronger association with OS, than changes in CA19–9. This is contrary to previous findings in a cediranib trial of patients with BTC, where IL6 stratified by changes of 10 pg/mL during treatment and after 3 months showed no prognostic association in the patient cohort as a whole (32). This discrepancy may be due to interpatient variability of circulating IL6 levels (e.g., the number of patients with elevated IL6 above the candidate threshold we reported in this study), or the cohort size (n = 118, 96 deaths observed), which is similar to our discovery cohort and may be underpowered. However, in contrast to the Backen and colleagues' work, we analyzed exponential $\log_2$ changes of IL6 from baseline and showed that a higher threshold of IL6 may have clinical relevance. Consequently, Backen and colleagues might not have detected a significant association of the full cohort due to testing of smaller changes of IL6. Finally, the ABC-03 trial was randomizing patients to standard gemcitabine and cisplatin in combination with placebo or cediranib (tyrosine kinase inhibitor of VEGF1/2/3). It is likely that VEGF inhibition may have an impact on IL6, because the placebo arm (n = 59) did show an association between IL6 and PFS and OS (PFS: HR, 1.04;95% CI, 1.02–1.07 and OS: HR, 1.02; 95% CI, 1.00–1.05), despite the above-mentioned limitations. As such, this warrants further investigation in an independent prospective trial.

Targeting tumor-promoting inflammation by inhibiting IL6 and YKL-40 signaling has been proposed as a therapeutic strategy by many groups (13, 17, 18, 23, 49, 50), as has the use of prognostic markers as targets (37). We show that gemcitabine induces tumor epithelial-derived expression of *IL6*, which is also supported by evidence from a previous study (51) and thus, IL6 may negatively affect OS by contributing to emerging gemcitabine resistance. This is supported by our HuCCT-1res cell line model and our finding that serum IL6 was higher in mice treated with gemcitabine compared with the control group. *IL6* mRNA expression levels in mouse tumor tissues did not correlate with serum IL6, which is in accordance with observations in patients with colorectal cancer (52), suggesting that circulating IL6 is a paraneoplastic phenomenon of systemic inflammation.

Exploration of outstanding questions connected to the impact of IL6R targeting in complex malignant environments such as in premetastatic niche formation (11) and immunologic equilibrium (53) is needed. Several phase I and II clinical trials are ongoing in patients with cancer, evaluating standard chemotherapy in combination with either anti-IL6 or anti-IL6R therapy (13). Results from the ongoing PACTO study ("phase II study of the combination of nabpaclitaxel and gemcitabine with or without tocilizumab first-line in patients with advanced pancreatic cancer" NCT02767557) and BILTRACTO study ("a single center, randomized, phase II study of the combination of cisplatin and gemcitabine with or without tocilizumab, an IL6R inhibitor, as first-line treatment in patients with locally advanced or metastatic biliary tract cancer" EudraCT 2018–004826-27) are pending.

A limitation of our study is that all three biomarkers were not available in all patients and as such biomarker performance was not directly comparable and results should be considered explorative. Furthermore, because targeting IL6R was performed considering an assumed contribution to the emergence of gemcitabine resistance, we recognize that the holistic effects of targeting IL6R in patients cannot be deducted from our work.

In conclusion, we have shown that IL6 and YKL-40 provide clinically relevant prognostic information when used alone or in combination with CA19–9 across patients with heterogeneous BTC. IL6 appears to be prognostically superior to other markers in none-levated CA19–9 patients and in patients with early death (within 6 months). IL6 and YKL-40 provide information that is independent of tumor location or treatment. Changes in IL6 during chemotherapy also provide independent information regarding OS and may be superior to changes in CA19–9. Finally, our data suggest that IL6R is a potential therapeutic target in the context of augmenting gemcitabine response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Disclosure of Potential Conflicts of Interest

D. Høgdall reports grants from Lundbeck Foundation (R180–2014-2999), Fabrikant Ejner Willumsens Memorial Foundation, Roche Denmark A/S, and Danish Society for Clinical Oncology during the conduct of the study. L.H. Jensen reports other from MSD (institution fee for clinical trial), Incyte (institution fee for clinical trial), 2cureX (institution fee for clinical trial), and BMS (institution fee for clinical trial) outside the submitted work. M. Mau-Sørensen reports other from Genmab (spouse employment and holder of stocks), personal fees from Roche (advisory board), Genmab (advisory board), and Bayer (advisory board), and grants from Karyopharm (research funding) and PUMA Biotechnologies (research funding) outside the submitted work. J.B. Andersen reports non-financial support from SEALD outside the submitted work. No potential conflicts of interest were disclosed by the other authors.

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Translational Relevance

Clinical management of biliary tract cancer is challenging because of limited treatment options and rapid emergence of chemoresistance, ultimately resulting in poor prognosis for patients, with a median overall survival less than 12 months. Serum CA19–9 is the most investigated prognostic biomarker in these patients. However, approximately 10% of the population do not produce CA19–9, rendering this marker prognostically uninformative in a substantial proportion of patients. It is important to explore novel prognostic biomarkers and therapeutic strategies that may guide clinicians toward better management. Here, we investigate the prognostic value of the circulating inflammatory biomarkers, IL6 and YKL-40, as well as the potential therapeutic benefit of targeting IL6R. The results suggest that serum IL6 and YKL-40, before and during treatment, provide new prognostic information either alone or in combination with CA19–9, ultimately improving patient stratification and early allocation to experimental protocols. Moreover, IL6R inhibitors may be considered in combination with gemcitabine and cisplatin.

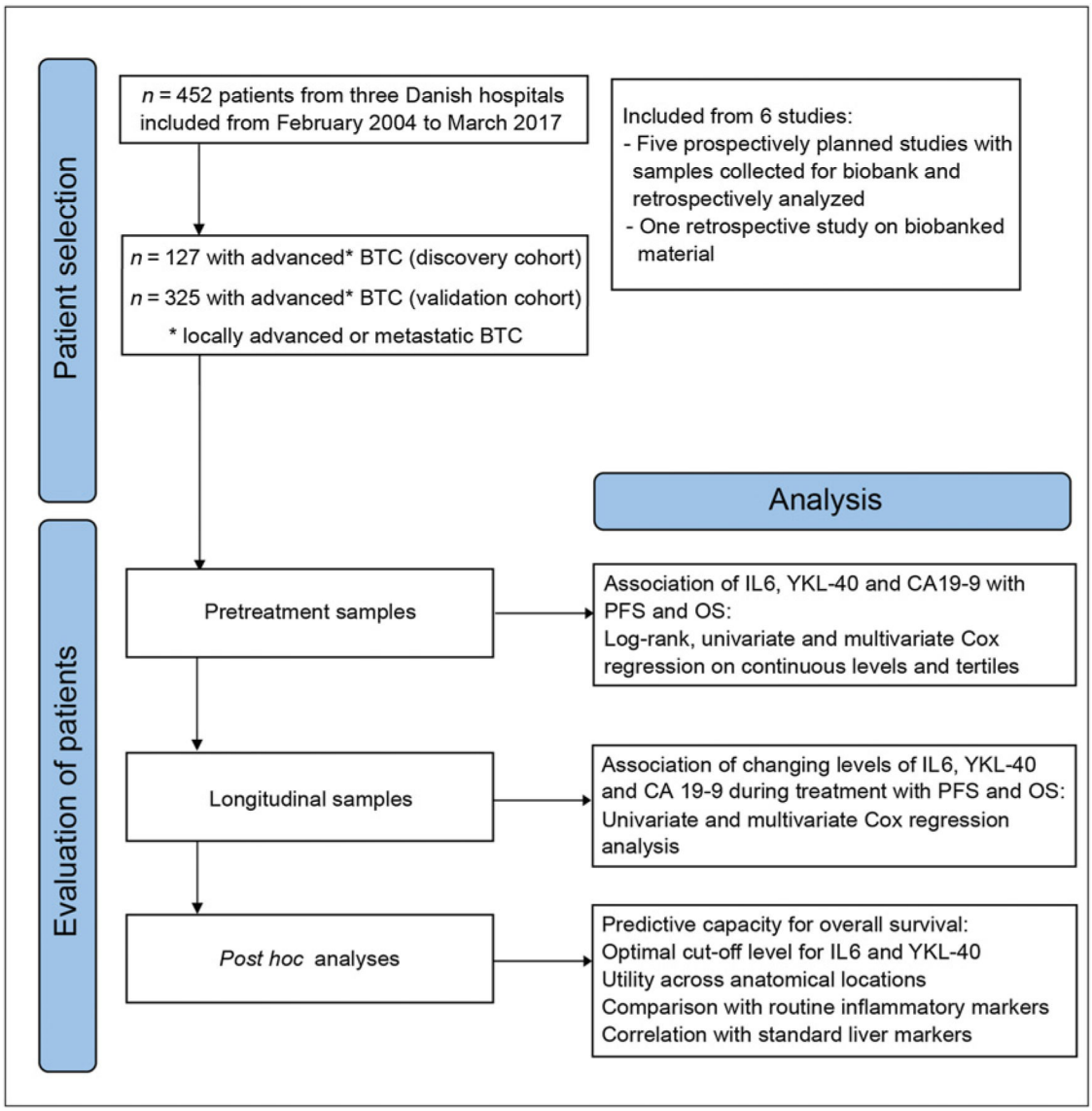

Figure 1.
Overview of BTC patient inclusion and workflow of analyses.

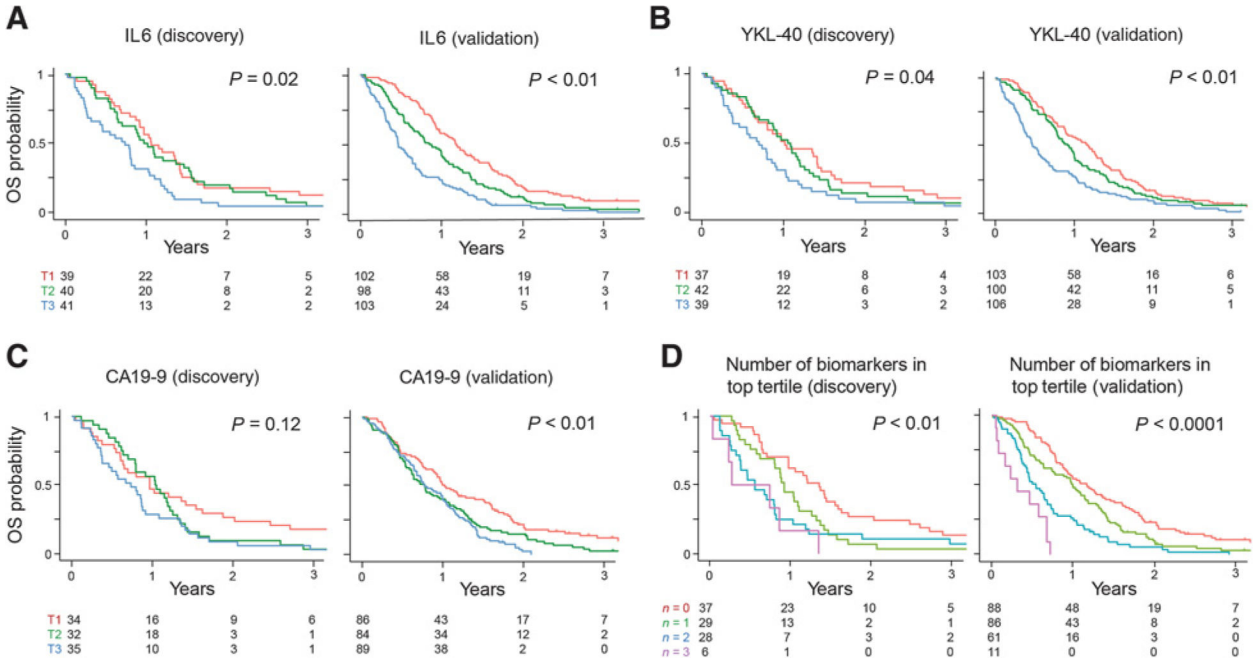

Figure 2.

Association of serum IL6, YKL-40, and CA19–9 with OS of patients with BTC. Kaplan-Meier plots associating BTC patient OS with pretreatment serum levels of IL6 (**A**), YKL-40 (**B**), CA19–9 (**C**), and number of biomarkers in the highest tertile (**D**). *P* values in Kaplan–Meier plots are based on a log-rank analysis. T, tertile; $n = 0$, no biomarkers in tertile 3; $n = 1$, one biomarker in tertile 3; $n = 2$, two biomarkers in tertile 3; and $n = 3$, three biomarkers in tertile 3.

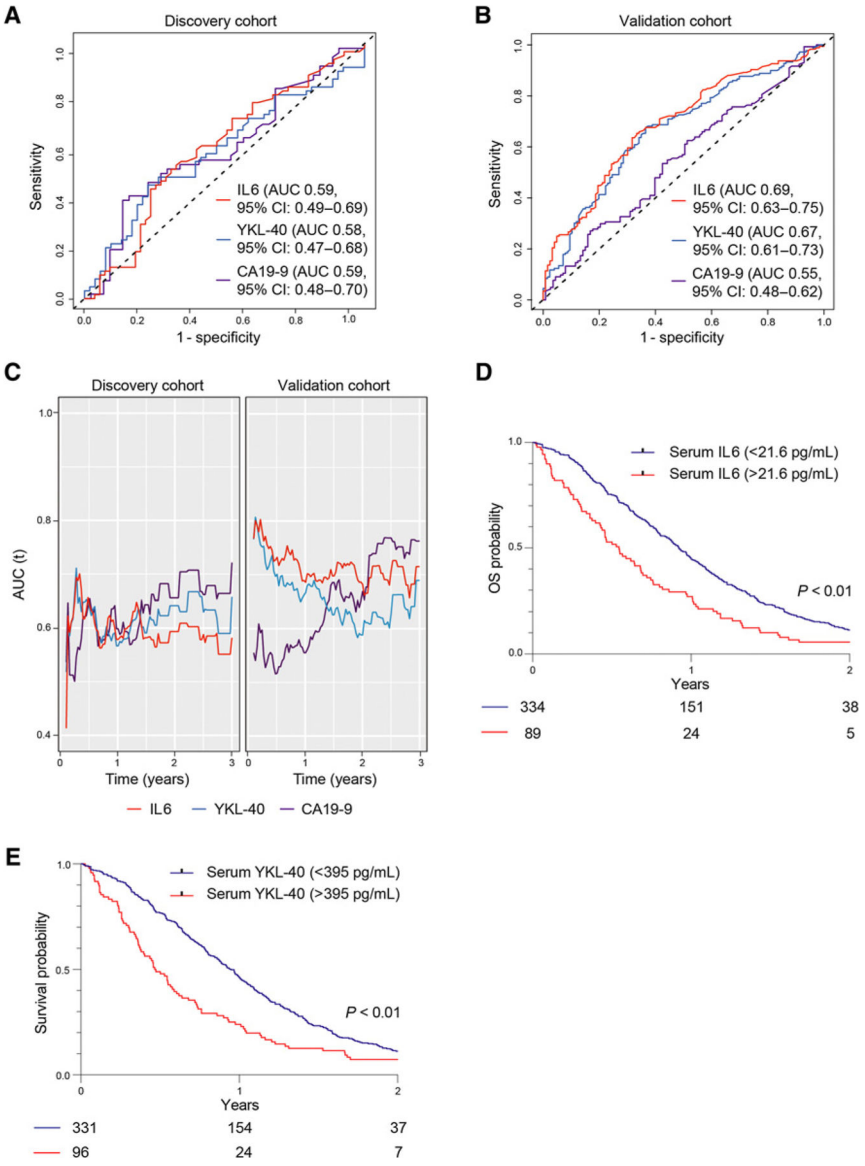

Figure 3.

Association of serum IL6 and YKL-40 with BTC patient OS stratified by survival time and using novel BTC-specific thresholds for elevation. **A** and **B,** ROC curves comparing the potential of serum IL6, YKL-40, and CA19–9 to predict survival beyond 12 months in discovery and validation cohorts. **C,** Continuous AUC plot for predicting OS at all timepoints until 3 years. **D,** IL6 elevation (defined by a novel BTC-specific threshold) and OS in pooled advanced BTC patient cohort. **E,** YKL-40 elevation (defined by a novel BTC-specific threshold) and OS in pooled advanced BTC patient cohort.

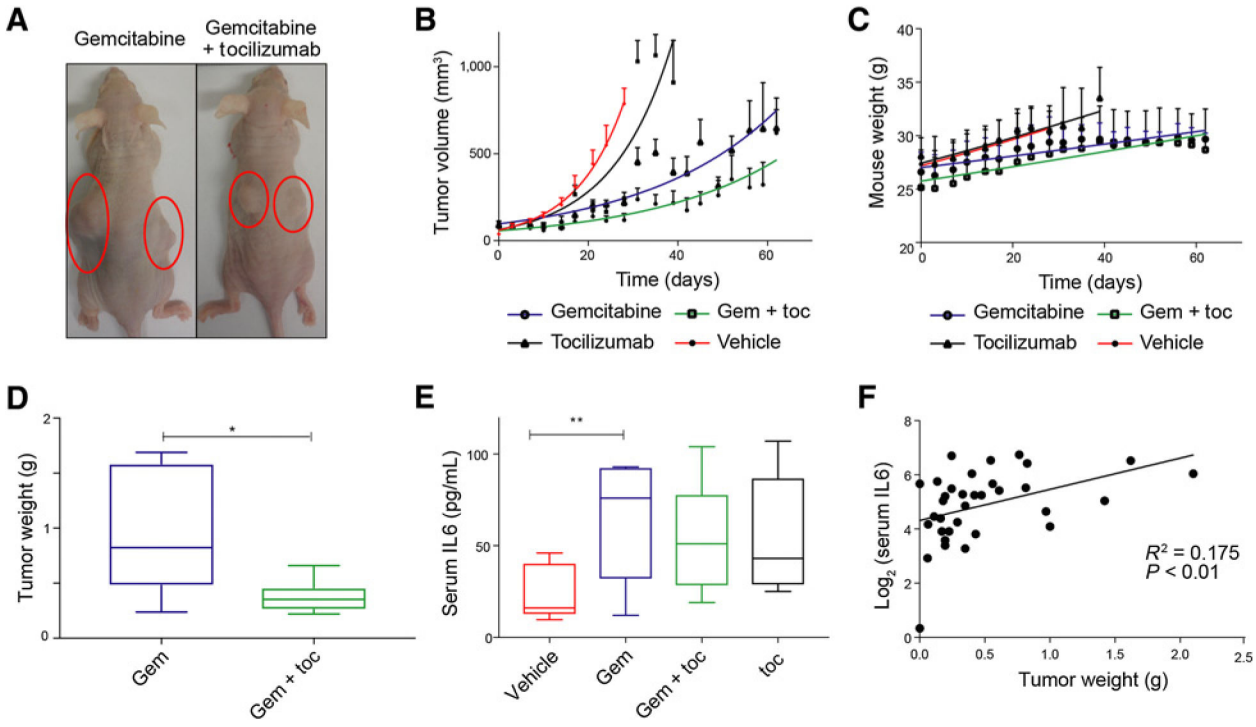

Figure 4.

Evaluating the therapeutic potential of tocilizumab (toc, anti-IL6R) *in vivo* in a BTC xenograft model. **A**, Representative images of mice treated with gemcitabine (Gem) and gemcitabine incombinationwith tocilizumab. **B,** Tumor growth rate (quantified twice weekly) over time across treatment groups. Error bars, SD. **C,** Mouse weight (quantified weekly) over time across treatment groups. Error bars, SD. **D,** Tumor weight at sacrifice between gemcitabine and gemcitabine + tocilizumab treatment groups.*, $P < 0.05$. **E,** Quantification of serum IL6 across treatment groups.**, $P < 0.01$. **F,** Correlation between serum IL6 and tumor weight.

Table 1.

Clinical characteristics of patients with BTC in discovery and validation cohorts.

	Discovery cohort	Validation cohort	*P*
Number of patients	127	325	
Age, median (range)	67 (36–84)	64 (21–83)	0.07
Sex, male number (%)	45 (35.4%)	140 (43.1%)	0.17
Localization			
Intrahepatic	44 (34.6%)	131 (40.3%)	<0.01
Perihilar	23 (18.1%)	25 (7.7%)	
Distal	37 (29.1%)	80 (24.6%)	
Gallbladder	14 (11.0%)	29 (8.9%)	
BTC[a]	9 (7.1%)	60 (18.5%)	
Metastasis			
None/locally advanced	31 (24.4%)	47 (14.5%)	<0.01
Yes	86 (67.7%)	262 (80.6%)	
Lymph nodes[b]	10 (7.9%)	11 (3.4%)	
Unknown	0 (0%)	5 (1.5%)	
PS (ECOG)			
0	68 (53.5%)	117 (36.0%)	<0.01
1	47 (37.0%)	138 (42.5%)	
2	12 (9.4%)	52 (16.0%)	
Unknown	0 (0%)	18 (5.5%)	
Treatment			
GCOC	56 (44.1%)	0 (0 %)	<0.01
GCO + BP	71 (55.9%)	72 (22.2%)	
GCO	0 (0%)	182 (56.0%)	
GC	0 (0%)	41 (12.6%)	
GCIB	0 (0%)	30 (9.2%)	
Survival			
PFS months, median (95% CI)	9.8 (8.5–12.2)	7.8 (7.0–8.8)	<0.01

	Discovery cohort	Validation cohort	*P*
OS months, median (95% CI)	11.2 (9.7–13.3)	10.5 (9.1–11.9)	0.01
Biomarkers			
CA 19–9 U/mL, median (range)	216 (3–846,000)	182 (3–121,000)	0.57
CA 19–9 elevated, number (%)	81 (63.8%)	204 (62.8%)	0.78
IL6 pg/mL, median (range)	7.7 (1.4–144)	8.2 (0.6–130)	0.12
IL6 elevated, number (%)	83 (65.4%)	228 (70.2%)	0.05
YKL-40 ng/mL, median (range)	151 (20–1,450)	185 (22–5,968)	0.12
YKL-40 elevated, number (%)	54 (42.5%)	171 (52.6%)	0.08

Abbreviations: ECOG, Eastern Cooperative Oncology Group; GC, gemcitabine and cisplatin; GCIB, gemcitabine, capecitabine, irinotecan, and bevacizumab; GCO + BP, gemcitabine, capecitabine, oxaliplatin, bevacizumab, and panitumumab; GCO, gemcitabine, capecitabine, and oxaliplatin; GCOC, gemcitabine, capecitabine, oxaliplatin, and cetuximab.

[a]Values are numbers (%).

[b]Lymph node metastases only.

Table 2.

Pretreatment univariate and multivariate Cox analysis between OS and clinical characteristics.

		Univariate Cox analysis				Multivariate Cox analysis log[a]				Multivariate Cox analysis tertiles			
		Discovery study		Validation study		Discovery cohort		Validation cohort		Discovery cohort		Validation cohort	
	Variable	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Age	Below median vs. above	0.73 (0.51–1.05)	0.09	1.00 (0.80–1.25)	1.00								
Sex	Male vs. female	0.88 (0.60–1.29)	0.52	1.16 (0.92–1.45)	0.21								
Location	Perihilar vs. IHC	0.88 (0.52–1.48)	0.63	0.74 (0.48–1.15)	0.19								
	Extrahepatic vs. IHC	1.06 (0.67–1.68)	0.80	0.92 (0.69–1.23)	0.58								
	Gallbladder vs. IHC	1.77 (0.96–3.25)	0.07	1.14 (0.75–1.74)	0.55								
	BTC[b] vs. IHC	0.91 (0.44–1.88)	0.80	0.92 (0.67–1.27)	0.63								
Metastasis	Yes vs. no	1.69 (1.09–2.61)	0.02	1.46 (1.05–2.02)	0.02	1.65 (0.99–2.76)	0.06			1.75 (1.03–2.99)	0.04		
	Lymph nodes[c] vs. no	1.20 (0.58–2.47)	0.63	1.39 (0.70–2.78)	0.35	1.07 (0.48–2.41)	0.87			1.00 (0.44–2.27)	1.00		
PS	1 vs. 0	1.01 (0.62–1.48)	0.97	1.80 (1.39–2.33)	<0.01			1.58 (1.16–2.16)	<0.01			1.68 (1.23–2.30)	<0.01
	2 vs. 0	1.60 (0.86–2.98)	0.14	2.42 (1.72–3.39)	<0.01			1.63 (1.09–2.43)	0.02			1.70 (1.13–2.55)	0.01
Treatment	GCOC vs. GCP + OP	0.78 (0.54–1.12)	0.18	NA	NA								
	GCO + BP vs. GC	NA		1.37 (0.91–2.06)	0.13								
	GCO vs. GC			1.14 (0.79–1.65)	0.48								
	GCIB vs. GC			1.63 (1.00–2.67)	0.05								
Biomarkers	CA 19–9 log[a]	1.06 (1.01–1.12)	0.02	1.08 (1.04–1.13)	<0.01	1.06 (1.00–1.11)	0.04	1.06 (1.02–1.11)	<0.01				
(continuous)	IL6 log[a]	1.11 (0.99–1.26)	0.08	1.36 (1.26–1.48)	<0.01	1.01 (0.86–1.19)	0.91	1.25 (1.12–1.38)	<0.01				
	YKL-40 log[a]	1.13 (0.98–1.30)	0.10	1.25 (1.15–1.36)	<0.01	1.19 (0.98–1.44)	0.08	1.11 (0.99–1.24)	0.08				
Biomarkers	CA 19–9 T2 vs. T1	1.16 (0.70–1.90)	0.56	1.52 (1.11–2.09)	<0.01					1.00 (0.60–1.68)	0.99	1.44 (1.03–2.02)	0.03
(tertiles)	CA 19–9 T3 vs. T1	1.75 (1.07–2.85)	0.03	1.86 (1.35–2.55)	<0.01					1.53 (0.92–2.56)	0.10	1.88 (1.32–2.68)	<0.01

Variable	Univariate Cox analysis				Multivariate Cox analysis log[a]				Multivariate Cox analysis tertiles			
	Discovery study		Validation study		Discovery cohort		Validation cohort		Discovery cohort		Validation cohort	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
IL-6 T2 vs. T1	1.07 (0.68–1.68)	0.76	1.55 (1.16–2.07)	<0.01					0.76 (0.45–1.29)	0.31	1.40 (1.00–1.94)	<0.05
IL-6 T3 vs. T1	1.94 (1.24–3.04)	<0.01	2.63 (1.89–3.37)	<0.01					1.11 (0.62–2.00)	0.73	2.05 (1.44–2.91)	<0.01
YKL-40 T2 vs. T1	1.31 (0.83–2.07)	0.25	1.19 (0.90–1.57)	0.23					0.30 (0.75–2.26)	0.36	0.81 (0.57–1.14)	0.22
YKL-40 T3 vs. T1	1.84 (1.16–2.92)	<0.01	1.93 (1.46–2.55)	<0.01					1.90 (1.02–3.55)	0.04	1.41 (1.01–1.98)	0.04

Note: Multivariate analysis was performed for all significant clinical characteristics (discovery cohort, metastasis and validation cohort, PS 1 and 2).

Abbreviations: GC, gemcitabine and cisplatin; GCIB, gemcitabine, capecitabine, irinotecan, and bevacizumab; GCO + BP, gemcitabine, capecitabine, oxaliplatin, bevacizumab, and panitumumab; GCO, gemcitabine, capecitabine, and oxaliplatin; GCOC, gemcitabine, capecitabine, oxaliplatin, and cetuximab; IHC, intrahepatic cholangiocarcinoma.

[a]All biomarkers are included as log values.

[b]No data on anatomic origin of BTC.

[c]Lymph nodes metastases only.

Table 3.

Univariate and multivariate Cox analysis of the association between time-varying changes from pretreatment levels of serum CA19–9, IL6, and YKL-40 and PFS and OS during chemotherapy.

		Univariate Cox analysis				Multivariate Cox analysis			
		Discovery study		Validation study		Discovery study		Validation study	
	Biomarkers	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
	Metastasis (yes vs. no)					1.59 (0.79–3.23)	0.20	1.71 (0.72–4.03)	0.22
	Lymph nodes** vs. no					1.26 (0.47–3.34)	0.65	1.72 (0.55–5.32)	0.35
	PS (1 vs. 0)							1.16 (0.70–1.90)	0.57
	PS (2 vs. 0)							1.19 (0.54–2.64)	0.66
PFS	Treatment GCO + BP vs. GC							2.63 (0.99–6.98)	0.05
	Treatment GCIB vs. GC							1.26 (0.53–3.00)	0.60
	Treatment GCO vs. GC							1.33 (0.56–3.18)	0.52
	CA 19–9	1.14 (1.02–1.27)	0.02	1.03 (0.98–1.08)	0.28	1.07 (0.95–1.21)	0.27	1.03 (0.97–1.10)	0.35
	IL6	1.33 (1.14–1.54)	<0.01	1.29 (1.14–1.47)	<0.01	1.30 (0.96–1.77)	0.09	1.19 (1.01–1.41)	0.04
	YKL-40	1.19 (1.02–1.39)	0.02	1.25 (1.09–1.43)	<0.01	1.24 (0.89–1.72)	0.21	1.11 (0.92–1.35)	0.27
	Metastasis (yes vs. no)					1.49 (0.69–3.21)	0.31		
	Lymph nodes** vs. no					0.79 (0.28–2.22)	0.66		
OS	PS (1 vs. 0)							1.59 (1.00–2.55)	0.05
	PS (2 vs. 0)							1.92 (1.01–3.66)	<0.05
	CA 19–9	1.23 (1.11–1.36)	<0.01	1.07 (1.02–1.13)	<0.01	1.16 (1.02–1.31)	0.02	1.06 (0.99–1.13)	0.08
	IL6	1.38 (1.20–1.60)	<0.01	1.38 (1.21–1.58)	<0.01	1.41 (1.03–1.93)	0.03	1.25 (1.05–1.49)	0.01
	YKL-40	1.25 (1.07–1.47)	<0.01	1.27 (1.09–1.47)	<0.01	1.20 (0.87–1.66)	0.26	1.11 (0.92–1.33)	0.30

Note: Multivariate analyses were performed using all univariately significant clinical characteristics (PFS: discovery cohort, metastasis and validation cohort, metastasis, PS 1 and 2, and treatment; and OS: discovery cohort, metastasis and validation cohort: PS 1 and 2). All biomarkers were included as log-transformed values and results are presented as HR per $\log_2$ change (doubling) from baseline.

Abbreviations: GC, gemcitabine and cisplatin; GCIB, gemcitabine, capecitabine, irinotecan, and bevacizumab; GCO + BP, gemcitabine, capecitabine, oxaliplatin, bevacizumab, and panitumumab.