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ORIGINAL ARTICLE

Bcl-x_L as prognostic marker and potential therapeutic target in cholangiocarcinoma

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Abstract

Intrahepatic, perihilar, and distal cholangiocarcinoma (iCCA, pCCA, dCCA) are highly malignant tumours with increasing mortality rates due to therapy resistances. Among the mechanisms mediating resistance, overexpression of anti-apoptotic Bcl-2 proteins (Bcl-2, Bcl-x_L, Mcl-1) is particularly important. In this study, we investigated whether antiapoptotic protein patterns are prognostically relevant and potential therapeutic targets in CCA. Bcl-2 proteins were analysed in a pan-cancer cohort from the NCT/DKFZ/DKTK MASTER registry trial (n = 1140, CCA n = 72) via RNA-sequencing and transcriptome-based protein activity interference revealing high ranks of CCA for Bcl-x_L and Mcl-1. Expression of Bcl-x_L, Mcl-1, and Bcl-2 was assessed in human CCA tissue and cell lines compared with cholangiocytes by immunohistochemistry, immunoblotting, and quantitative-RT-PCR. Immunohistochemistry confirmed the upregulation of Bcl-x_L and Mcl-1 in iCCA tissues. Cell death of CCA cell lines upon treatment with specific small molecule inhibitors of Bcl-x_L (Wehi-539), of Mcl-1 (S63845), and Bcl-2 (ABT-199), either alone, in combination with each other or together with chemotherapeutics was assessed by flow cytometry. Targeting Bcl-x_L induced cell death and augmented the effect of chemotherapy in CCA cells. Combined inhibition of Bcl-x_L and Mcl-1 led to a synergistic increase in cell death in CCA cell lines. Correlation between Bcl-2 protein expression and survival was analysed within three independent patient cohorts from cancer centers in Germany comprising 656 CCA cases indicating a prognostic value of Bcl-x_L in CCA depending on the CCA subtype. Collectively, these observations identify Bcl-x_L as a key protein in cell death resistance of CCA and may pave the way for clinical application.

KEYWORDS
apoptosis, Bcl-2, Bcl-x_L, chemotherapy, cholangiocarcinoma, Mcl-1

1 | INTRODUCTION

Cholangiocarcinoma (CCA) is a highly lethal cancer with an increasing incidence and mortality in the last decade.[1,2] Five-year survival does not exceed 10% for neither intrahepatic (iCCA) nor extrahepatic (eCCA) cholangiocarcinoma.[2,3] Due to late onset of symptoms, most patients are diagnosed at late stages of the disease making them non-eligible for potentially curative surgery. Even in potentially resectable cases, the prognosis remains poor.[4] Conventional chemotherapy shows limited efficacy in CCA, and targeted therapies are only available for a minority of patients.[5] Thus, it is of particular importance to develop new effective and less toxic treatment strategies to improve the patients' outcome.

Tumour cells acquire a variety of different survival mechanisms, among others avoidance of cell death leading to therapeutic resistance.[6] The B-cell lymphoma 2 (Bcl-2) family is commonly known for its crucial role in regulation of apoptosis.[7] Its antiapoptotic members such as Bcl-2, Bcl-x_L (B-cell lymphoma extra-large), and Mcl-1 (myeloid cell leukaemia sequence 1) are overexpressed in different tumour entities and can be targeted by BH3 (Bcl-2 homology) mimetic agents.[8,9] This class of molecules binds and inhibits the antiapoptotic Bcl-2 family members, thereby releasing proapoptotic proteins from their binding and initiating the intrinsic downstream apoptotic cascade.[10,11] The specific Bcl-2 inhibitor ABT-199 has received FDA-approval for treatment of chronic lymphocytic leukaemia (CLL) and acute myeloid leukaemia (AML).[12–14] In solid tumours, some BH3-mimetics showed promising signs of efficacy in preclinical studies either alone or in combination with standard-of-care therapies.[15–17]

Here, we aimed to assess the role of Bcl-2 family members in CCA by analysing their expression and association between transcriptional patterns and survival data in three large CCA cohorts including 280 iCCA and 376 eCCA cases, further subclassified into perihilar cholangiocarcinoma (pCCA) and distal CCA (dCCA). Furthermore, we investigated whether targeting antiapoptotic proteins with specific small molecule inhibitors in vitro could provide a new therapeutic strategy in CCA. Our results indicate the dependency of CCA on Bcl-x_L and identify its potential as candidate drug target.

Key points

In cholangiocarcinoma (CCA), a rare liver malignancy arising from bile duct cells, proteins of interest (Bcl-x_L and Mcl-1) appeared to be especially overexpressed compared with other malignant tumours and compared to normal liver tissue. Targeting those proteins via specific inhibitory molecules induced cell death and increased the impact of chemotherapy in CCA cells. Correlation of survival data with expression of these proteins in three large groups of CCA patients indicates that patients with tumours that show a higher expression of Bcl-x_L live longer than those with lower Bcl-x_L levels.

2 | MATERIAL AND METHODS

2.1 | RNA sequencing and estimation of protein activity in the NCT/DKTK MASTER cohort

The MASTER (Molecularly Aided Stratification for Tumour Eradication Research) trial of the NCT Heidelberg and the German Cancer Consortium (DKTK) is a multicenter, prospective observational study based on comprehensive molecular diagnostics, therapeutic decision-making, and structured follow-up. The inclusion criteria comprise younger patients (<51 years) or patients with rare tumour entities. The multi-omic workup includes whole-genome sequencing (WGS) or whole-exome sequencing (WES) and RNA sequencing.[18] The MASTER RNA-seq cohort used in this analysis comprised 1140 cases, including 46 cases with intrahepatic CCA and 26 cases with extrahepatic CCA. Before sequencing, all samples underwent quality control and estimation of tumour cell content by an experienced pathologist. Gene-level normalized RNA-seq data [tpm (transcripts per million) values] were used. Protein activity was estimated from the tpm values using the metaVIPER algorithm as previously described.[19,20]

2.2 | Tissue Micro-Array and Immunohistochemistry

The first Tissue Micro Array (HD-TMA), provided by the Tissue Bank of the National Center for Tumour Diseases (NCT, Heidelberg, Germany), consisted of 154 iCCA, 155 pCCA, and 126 dCCA cases. The second TMA (MUC-TMA) was constructed at the Institute of Pathology of the University of Munich (LMU) and comprises the tumour material of 28 iCCA and 26 eCCA patients. Each patient is represented by two respectively three allied spots (TMA cores). For most patients, complete clinicopathological data, including sex, age, grading, TNM/UICC status, as well as overall survival data were available. Study protocols received Institutional Review Board approval from the Ethics Committee (Medical Faculty of Heidelberg University, reference number: S-207/2015 and S-519/2019, LMU Munich 285–15 2).

Furthermore, we obtained paraffin-embedded iCCA (n = 10) and healthy liver tissue specimens (n = 10) from the Tissue bank of the NCT. The sections were deparaffinized and rehydrated by incubation in xylene and a series of graded alcohols, followed by antigen retrieval and staining with an antibody against Bcl-x_L (#2764, Cell Signalling Technology), Bcl-2 (Sigma SAB450003) and Mcl-1 (HPA031125) using the NovoLink Polymer Detection System (Leica Microsystems), according to the manufacturer's protocol.

Staining intensity was independently evaluated by two experienced examiners by utilizing a scoring system in which values for staining quantity (no expression = 0; <1% = 1; 1%–9% = 2; 10%–50% = 3; >50% = 4) and quality (negative = 0; low = 1; moderate = 2; strong = 3) were allocated and multiplied in the end as described

previously.[21] Negative controls were generated by omitting the primary antibody. Scores greater than or equal to the median were considered as "High expression", whereas scores lower than the median were considered as "Low expression".[22]

2.3 | Cell lines and cell culture

Human iCCA cell lines HUCCT1 and SNU1079 as well as eCCA cell lines SNU478 and SNU1196 were purchased from ATCC (Virginia, USA). Primary cultures of normal human cholangiocytes (NHC) were isolated as described by Banales et al.[23] CCA cell lines were maintained in RPMI 1640 (Gibco) supplemented with 10% Fetal Calf Serum (Pan Biotech) and 1% Penicillin/Streptomycin (Sigma-Aldrich) and cultured at 37°C in the presence of 5% CO_2. NHC cells were cultured in individually enriched DMEM F12 as previously described (Gibco).[24]

2.4 | Inhibitors and antibodies

ABT-199 was kindly provided by AbbVie, S63845 and Wehi-539 were purchased from Selleckchem and Cayman Chemicals, respectively. Inhibitors were dissolved in Dimethyl sulfoxide (DMSO) at a stock concentration of 10 μM and stored at −80°C until use. Antibodies used for Western blotting were the following: Bcl-x_L (#2764, Cell Signalling Technology), Mcl-1 (#ab28147, Abcam), Bcl-2 (#ab692, Abcam), Cyclin-D1 (#ab226977, Abcam), and Tubulin (#T8203, Sigma-Aldrich) as well as peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology).

2.5 | RNA extraction and cDNA synthesis

After designated treatments, total RNA was extracted from cells using RNeasy Plus Mini Kit from QIAGEN according to the instruction. cDNA was synthesized from 1000 ng mRNA using High-Capacity cDNA Reverse Transcription Kits (TaKaRA) following the manufacturer's protocol. Reverse transcription was carried out using a DNA thermal cycler (Bio-Rad). The thermal conditions were 37°C for 15 min and 85°C for 5 s, and cDNA was stored at −80°C.

2.6 | Real-time PCR and relative quantification of mRNA expression

The samples were prepared for rt-PCR using QuantiTect Primer Assays and the QuantiTect SYBR-Green PCR Kit from QIAGEN according to the instruction and transferred onto a 96-well plate. All measurements were done in duplicates using the LightCycler 480 from Roche. The mRNA expression was normalized to the expression of GAPDH as a house-keeping reference. Data analysis was done by using the Light Cycler 480 SW 1.5 software.

2.7 | Immunoblot analysis

Cell lysis and subsequent protein isolation were performed according to standard procedures using RIPA-lysis buffer. Proteins were separated by 12% SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes following standard procedures, and finally analysed by immunoblotting.

2.8 | Flow cytometry-based Cell Death Assay

Cells were seeded onto a 12-well plate and incubated at 37°C, 5% CO_2 for 24 h. The desired treatment was applied the day after seeding, the plates were once more incubated for 48 h. In order to harvest the treated cells, the supernatant from each well was transferred to a FACS tube each. Subsequently, the cell layers were harvested using Trypsin (PAA Laboratories) and pooled with the earlier removed cell medium in the tubes. After centrifugation (500× *g*, 5 min), the cell pellets were resuspended in Nicoletti-buffer containing 0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X-100 and 50 μg/mL propidium iodide (all from Sigma-Aldrich), vortexed thoroughly and incubated for 1 h in the dark at 4°C. FACS analysis was performed according to the protocol of Nicoletti et al. by FACS analysis, using the FACS Diva 6 and the FlowJo 7.6.5. software (BD Biosciences). Cells in the sub-G1 fraction were considered apoptotic.[25]

2.9 | Statistical analysis

Statistical Analysis was performed using GraphPad PRISM® 8.2. (GraphPad) and R software (©The R Foundation, www.r-project.com, Version 4.0.3). All in vitro experiments were performed in at least triplicates and data are presented as mean + SD. Obtained data were submitted to analysis of variance (ANOVA) with the post hoc Dunnett's multiple comparison test or by two-way repeated measures analysis of variance (two-way ANOVA) test followed by a post hoc Tukey's multiple comparison test. Non-parametric data, like the grading of an immunohistochemical staining, were analysed by using either the Mann–Whitney *U* test (non-paired data) or the Wilcoxon-test (paired data). Correlation between Bcl-2, Mcl-1, and Bcl-x_L mRNA tpm values and protein activity data was estimated using Pearson's correlation analysis. Association between protein expression and clinicopathological characteristics was assessed as a multivariate analysis using the Cox proportional hazard models. Survival curves were estimated by the Kaplan–Meier method and compared by log-rank test. Overall survival was calculated from first tumour diagnosis to the date of death or last follow-up. The best cut-point of mRNA tpm values to stratify cases into two groups for OS prediction was assessed using maximization of log-rank statistics.[26] Statistical significance was set to a two-tailed 0.05 *p*-value and is indicated as $^{***}p < .001$; $^{**}p < .01$; $^{*}p < .05$.

3 | RESULTS

3.1 | Bcl-x_L is upregulated and highly active in human cholangiocarcinoma tumours

We used a pan-cancer cohort (n = 1140) from the NCT/DKTK MASTER national multi-center registry trial to analyse the transcriptional pattern of the anti-apoptotic proteins Bcl-2, Bcl-x_L, and Mcl-1. The cohort included cases of eCCA (n = 26) and iCCA (n = 46, Table 1, Table S1). Since analysis of RNA sequencing data did not allow identification of posttranslational changes but only detected transcriptional changes, we applied the metaVIPER algorithm to estimate protein activity. This algorithm uses an integrative analysis of the expression of transcriptional targets of a gene of interest to assess its protein activity.[20]

Among all explored malignancies within the cohort, iCCA and eCCA had the highest protein activities of Bcl-x_L as well as one of the highest mRNA expression levels of *BCL2L1* (the gene encoding Bcl-x_L) (Figure 1A,B). In contrast, mRNA levels and estimated protein activity of Bcl-2 were relatively low in CCA compared with the other entities within the MASTER cohort. Interestingly, in all cases, the ranks of mRNA expression and protein activity did not match perfectly – especially, Mcl-1 showed discrepancies between its ranks possibly indicating a posttranslational effect. In iCCA, the rank of *MCL1* mRNA expression was much higher than the respective rank of Mcl-1 protein activity whereas in eCCA the exact opposite could be observed, pointing out biological differences between iCCA and eCCA. In order to confirm our observation, we performed immunohistochemistry staining for Bcl-x_L, Mcl-1, and Bcl-2 on representative tissue samples from the MASTER cohort. A high immunohistochemistry staining score of the examined proteins was correlated with higher mRNA expression levels as well as protein activity (Figure 1C, Figure S1).

TABLE 1 Case numbers and different characteristics of the three CCA cohorts. Case numbers of all three investigated CCA cohorts (HD-TMA, MASTER cohort, MUC-TMA) grouped according to the anatomical localization of the tumour and specific characteristics of the three different cohorts (median age, median UICC, resectability, and treatment with palliative therapy)

Cohort characteristics	HD-TMA	MASTER cohort	MUC-TMA
iCCA	154 (35%)	46 (64%)	28 (52%)
eCCA	281 (65%)	26 (36%)	26 (48%)
pCCA	155 (55%)		16 (62%)
dCCA	126 (45%)		10 (38%)
Median age	66	47	65
Q1; Q3	57; 73	38; 52	55; 71
Median UICC	II	IV	III
Sex			
Female	154 (35%)	28 (39%)	25 (46%)
Male	281 (65%)	44 (61%)	29 (54%)

Analyses of the expression data revealed that both *BCL2L1* and *MCL1* are highly expressed in contrast to a low *BCL2* expression (Figure S2e). This effect could be shown in all CCA patients as well as in the subgroups iCCA and eCCA (Figure S2f,g). Expression of all three genes showed a modest positive correlation (Figure 1E, Figure S2a,b. Remarkably, correlation of protein activity showed diverging results, namely a highly negative correlation for Bcl-2 and Bcl-x_L activity independent of the tumour localization. Mcl-1 and Bcl-2 activity showed no correlation, whereas Mcl-1 and Bcl-x_L showed a decent positive correlation in eCCA only (Figure 1D, Figure S2c,d).

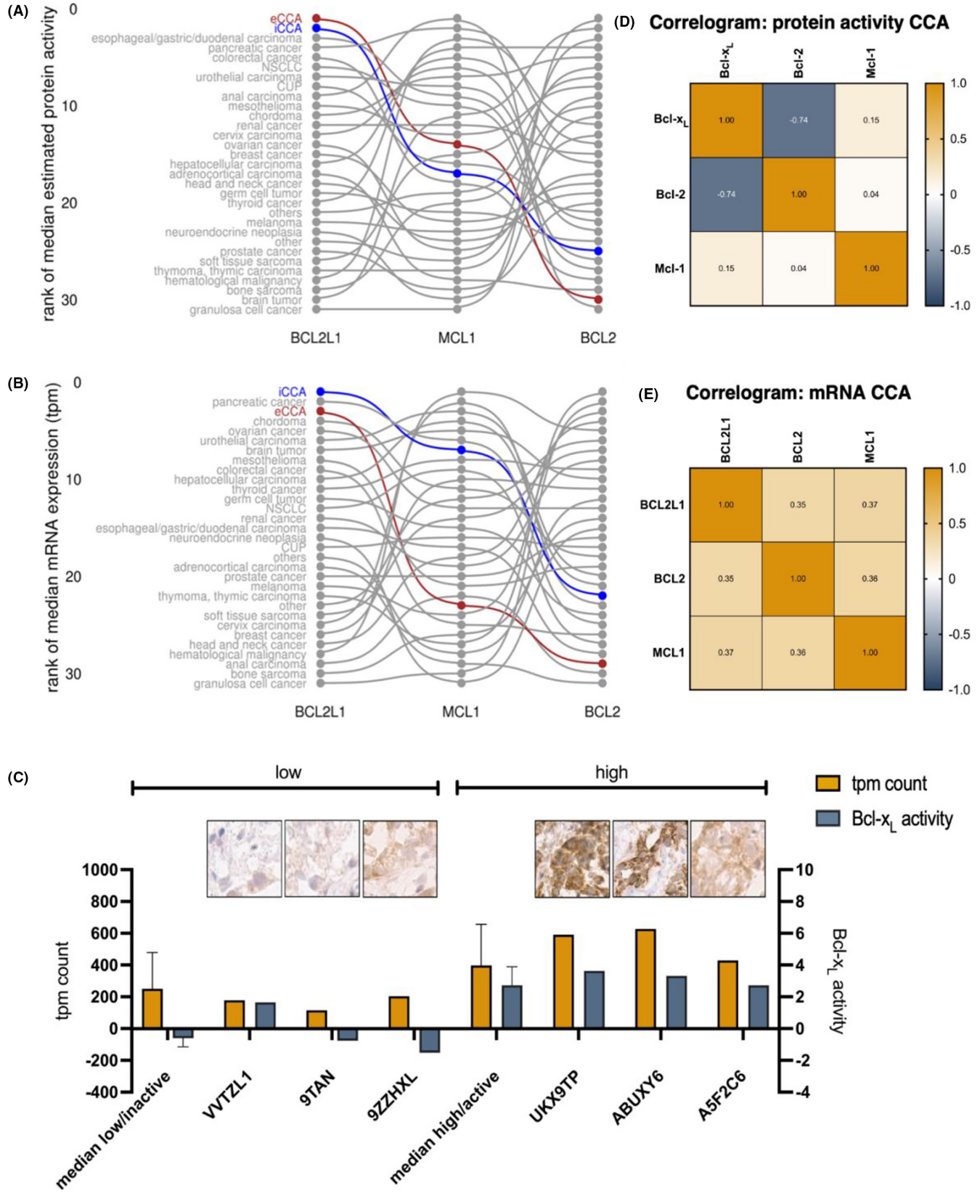
3.2 | Bcl-x_L and Mcl-1 are overexpressed in iCCA compared with cholangiocytes in non-neoplastic liver tissue

In order to validate the results from the NCT/DKTK MASTER registry, we examined Bcl-x_L, Mcl-1, and Bcl-2 protein expression in tissue samples (n = 10) of human iCCA via immunohistochemistry and compared them to protein expression in normal cholangiocytes on the same sample and normal liver tissue (n = 10), respectively. In iCCA, both Bcl-x_L and Mcl-1 were found to be significantly overexpressed compared with normal cholangiocytes on the same slide (Figure 2A,B). In comparison to cholangiocytes in non-neoplastic human liver samples, Bcl-x_L and Mcl-1 were also significantly overexpressed in iCCA (Figure 2C,D). In contrast, Bcl-2 did not show changes in expression in iCCA, neither compared with adjacent non-neoplastic liver tissue nor compared with normal human liver samples (Figure 2A–D).

3.3 | CCA cell lines overexpress antiapoptotic proteins and are sensitive to Bcl-x_L specific inhibition

For further investigation of the role of Bcl-2 proteins in CCA, we assessed the expression of Bcl-x_L, Bcl-2, and Mcl-1 in four human CCA cell lines (iCCA: HUCCT1, SNU1079; eCCA: SNU478, SNU1196) and normal human cholangiocytes (NHC) using rt-qPCR and Western immunoblotting (Figure 3A,B). Compared with NHC, Bcl-x_L was significantly overexpressed in both rt-qPCR and immunoblotting in three out of four employed cell lines compared with NHC. Mcl-1 was overexpressed in all cell lines in Western immunoblotting but did only show significant changes on RNA expression level in one cell line (Figure 3A,B) compared with non-transformed cholangiocytes. Bcl-2 showed low expression in all employed CCA cell lines and no expression in NHC.

To evaluate the efficacy of target-specific BH3-mimetics in inducing cell death, the four employed cell lines were incubated with Wehi-539 (Bcl-x_L inhibitor), ABT-199 (Bcl-2 inhibitor), and S63845 (Mcl-1 inhibitor) in seven different concentrations, and cell death was subsequently quantified by FACS analysis (Figure 3C). All tested cell lines were most sensitive towards inhibition of Bcl-x_L—the maximal concentration of Wehi-539 induced up to 40% of cell death.



However, the degree of cell death induction varied greatly between the different cell lines irrespective of the anatomic origin of the cell line. For further experiments, inhibitor concentrations were chosen that exceeded basal cell death by around 5%–10%.

Subsequent immunoblot analyses of the treated cell lines using antibodies against Bcl-x_L, Bcl-2, Mcl-1, and Cyclin D1 showed an up-regulation of Bcl-x_L (Figure 3D) and Bcl-2 (Figure 3E) under treatment with Wehi-539. Mcl-1 did not show any changes under therapy.

FIGURE 1 Bcl-x_L, Bcl-2 and Mcl-1 in a pan-cancer patient cohort. Rank of median estimated protein activity (A) and mRNA expression (B) for antiapoptotic proteins Bcl-x_L, Bcl-2 and Mcl-1 in a pan-cancer patient cohort from the DKTK/MASTER registry trial (n = 1140). Normalized tpm expression values were used, and the protein activity estimated from RNA-seq data using the metaVIPER algorithm. The connected dots belong to the same entity. (C) Correlation of tpm-count and Bcl-x_L activity within the MASTER cohort as bar chart with representative values and IHC images of the respective case showing a higher staining intensity for cases with high tpm counts, as well as high protein activity. (D) Correlogram of protein activity of Mcl-1, Bcl-2, and Bcl-x_L showing negative correlation of Bcl-2 and Bcl-x_L r = −.74 (p = .0005), no correlation of Bcl-2 and Mcl-1 r = .038 (p = .749) and positive correlation of Bcl-x_L and Mcl-1 r = .151 (p = .205). (E) Correlogram of tpm values MCL1, BCL2, and BCL2L1 showing significant positive correlation of the three proteins: correlation BCL2 vs. MCL1 r = .362 (p = .002), BCL2 vs. BCL2L1 r = .349 (p = .003), MCL1 vs. BCL2L1 r = .373 (p = .001).

Cyclin D1 was not altered suggesting an unaffected cell cycle progression (Figure 3D,E).

3.4 | Treatments with Bcl-x_L inhibitor or Mcl-1 inhibitor increase efficacy of chemotherapy

To test the response under treatment with chemotherapeutic agents 5-fluorouracil (5-FU), Gemcitabine and Cisplatin, the most used chemotherapeutics for patients with advanced CCA, the four CCA cell lines were incubated with equal doses of the respective cytostatic drug and cell death was subsequently quantified by FACS analysis (Figure 4A–C). Since upregulation of antiapoptotic proteins is known to render cells resistant towards chemotherapy, we combined chemotherapy with specific inhibition of the different Bcl-2-family proteins. Inhibitor doses as well as cytostatic drug concentrations were individually adjusted to induce moderate cell death increase under mono-treatment. Bcl-x_L inhibition via treatment with Wehi-539 sensitized three out of four cell lines to treatment with 5-FU (Figure 4D), Cisplatin (Figure 4E), and Gemcitabine (Figure 4F) leading to a synergistic increase of cell death. Combining Mcl-1 inhibition and chemotherapeutic treatment also lead to significantly increased cell death levels in three out of four cell lines (5-FU and Cisplatin) and four cell lines (Gemcitabine) (Figure S3a–c) while the combination with Bcl-2 inhibition did not show any impact on the response to chemotherapeutic treatment (Figure S3d–f).

3.5 | Combined inhibition of Bcl-x_L and Mcl-1 synergically induces cell death

Since chemotherapeutic treatment can cause significant adverse effects and toxicity, a chemotherapy-free treatment option could be valuable for many patients. We designed an experiment combining all three inhibitors with each other in order to evaluate whether cell viability could be significantly affected by a double inhibition. Strikingly, targeting Bcl-x_L and Mcl-1 via a combination of Wehi-539 and S63845 led to up to ten times higher cell death than single treatment reaching >80% CCA cell killing in all four cell lines (Figure 5A). Combination of Bcl-2 and Mcl-1 inhibition also showed a synergistic effect (Figure 5C), indicating the important role of Mcl-1 as contributor to therapeutic resistance. Three out of four cell lines did also respond to combination of Bcl-2 and Bcl-x_L inhibition with increased cell death levels (Figure 5B).

3.6 | Bcl-x_L expression as a subtype specific prognostic factor in CCA

In order to assess whether expression of the Bcl-2 proteins could serve as a prognostic factor in CCA, immunohistochemical staining for Bcl-x_L, Bcl-2, and Mcl-1 was performed for three different cohorts (MASTER cohort, HD-TMA, MUC-TMA, Table 1, Figure 6A) and evaluated using a score considering quantity and quality of staining. After classification as "high expression" and "low expression" based on the scoring system described above, the results were correlated with survival data of the patients. Clinicopathological data and multivariate analysis are listed in Tables S1–S4. Within the HD-TMA cohort, age and TNM stage appeared to have an impact on survival (Tables S2–S5) whereas in the MASTER-cohort only TNM stage showed such an effect (Table S1). Multivariate analysis of clinicopathological data between iCCA, pCCA, and dCCA is listed in Table S5 revealing significant impact of TNM, histological grade, Bcl-x_L and Mcl-1 status on survival within the subgroups: High Bcl-x_L as well as high Mcl-1 status, TNM stages III-IV and a high histological grading (G3) is correlated with a worse outcome. Intriguingly, the HD-TMA showed significantly higher scores for Bcl-x_L expression for iCCA compared with pCCA and dCCA (Figure S5a–c). In all CCA cases as well as just in the pCCA subtype, high expression of Bcl-x_L correlated with a better overall survival of the patients (Figure 6B,D) whereas in iCCA and dCCA expression of Bcl-x_L did not have a significant impact on patients' outcome (Figure 6C,E). In all CCA cases and in the anatomic groups iCCA, pCCA, and dCCA, expression of Bcl-2 and Mcl-1 did not show any association with survival (Figure S4). Both proteins showed significantly higher staining scores in pCCA compared with dCCA and iCCA (Figure S5b,c).

When distinguishing small and large bile duct type within the iCCA subgroup, we found a tendency of better survival for the "small bile duct" subgroup (Figure S6a, 6.4 vs. 3.1 years, p = .1370). Analysis of the influence of Bcl-x_L status on survival in small vs. large bile duct type showed no difference in survival for only "large" or only "small" iCCA cases (Figure S6c,d). No significant impact was seen for Mcl-1 nor Bcl-2 status on survival in either small or large bile duct subgroups (data not shown). No difference was seen between the respective IHC scores between the subgroups (Figure S6b).

Validation of these observations using the MUC-TMA cohort showed that Bcl-x_L was a prognostic marker in CCA since high expression of the protein correlated significantly with a longer survival of the patients (Figure 6F). In a multivariate stepwise forward Cox regression analysis of the Munich cohort patients adjusting for age,

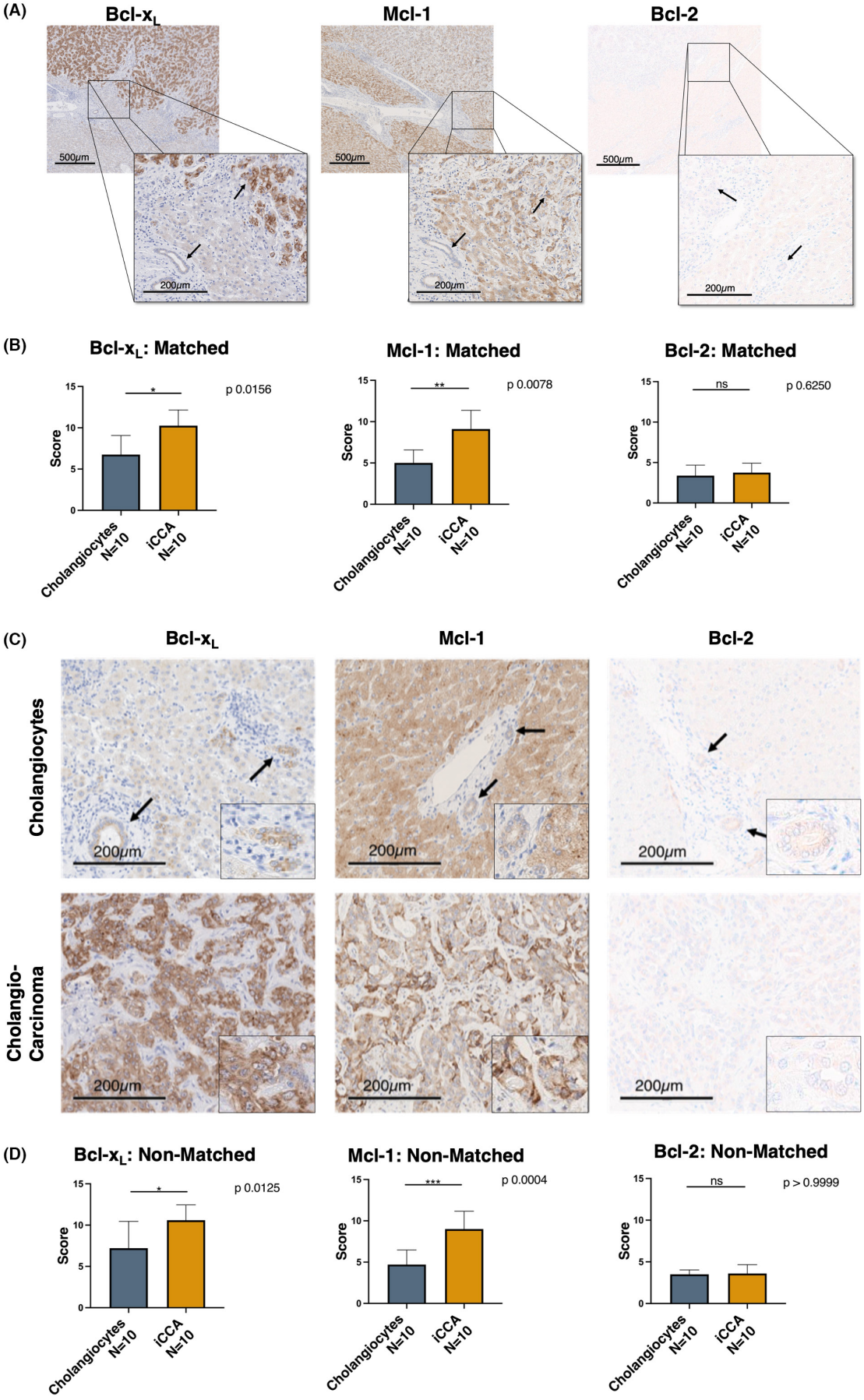


FIGURE 2 Bcl-x_L and Mcl-1 are upregulated in human cholangiocarcinoma. Immunohistochemical staining for Bcl-x_L, Mcl-1, and Bcl-2 respectively, performed on human cholangiocarcinoma tissue ($n = 10$) and adjacent non-tumor tissue (A) or non-neoplastic liver tissue ($n = 10$) (c). Representative images are shown. (B, D) Semi-quantitative analysis of staining intensity by multiplication of values for staining quantity and quality, showing significantly increased expression in CCA of both Mcl-1 and Bcl-x_L compared to adjacent non-tumor tissue (matched analysis, Bcl-x_L $p = .0156$, Mcl-1 $p = .0078$) (B) and non-neoplastic liver tissue (non-matched analysis, Bcl-x_L $p = .0125$, Mcl-1 $p = .0004$) (D) and no significant difference in Bcl-2 expression (matched analysis $p = .6250$; non-matched analysis $p > .9999$). Mann-Whitney test (B) and Wilcoxon test (D) were performed to compare the groups. Data is shown as mean ± SD

sex, tumour localization, and TNM-stage, Bcl-x_L expression turned out as sole independent prognosticator for OS ($p = .043$; HR 0.492; 95% CI: 0.248–0.978).

In order to test our findings in a cohort of heavily pretreated stage IV patients, the results of RNA sequencing of the MASTER cohort were correlated with their overall survival. Patients with high expression of BCL2L1 (tpm values >182) showed higher median overall survival in a multivariate model accounting for age, gender and tumour location (Figure 6G). No interaction was observed with primary localization of the tumour (iCCA vs eCCA), as the effect pointed to the same direction in both subgroups (Figure S5d,e).

4 | DISCUSSION

Patients diagnosed with advanced cholangiocarcinoma not eligible for curative surgery are facing limited treatment options with poor response rates.[3] For a minority of patients only, targeted therapies based on molecular stratifications are available.[27,28] Identifying means to either augment the efficiency and tolerability of standard chemotherapy or to discover new molecularly guided treatment options could proof essential to improve patients' outcome. Upregulation of antiapoptotic proteins and thus avoidance of apoptosis is crucial for primary and acquired therapeutic resistance.[6,15] In this study, we assessed the role of antiapoptotic Bcl-2 proteins in CCA regarding prognosis and therapeutic exploitation.

Predicting protein activity from RNA sequencing is a powerful tool to predict protein expression.[17,20] We used RNA sequencing data from a large pan-cancer cohort (NCT/DKTK MASTER cohort) to estimate protein activity of Bcl-x_L, Mcl-1, and Bcl-2. iCCA and eCCA showed the highest protein activity and high mRNA expression ranks for Bcl-x_L. Mcl-1 showed a distinct correlation between mRNA expression and protein activity depending on CCA subtype. Bcl-2 expression and activity were comparably low. These observations are in line with previous studies demonstrating overexpression of Bcl-x_L and Mcl-1 in cholangiocarcinoma. Expression of Bcl-2 has been reported inconsistently.[29–33] We observed slight differences between RNA expression and protein activity, whereas Mcl-1 showed the greatest distinction. Compared with Bcl-2 and Bcl-x_L, Mcl-1 has a rather short half-life and is subjected to specific and swift post-translational regulation possibly explaining different transcript levels and estimated Mcl-1 activity.[34,35] Post-translational regulation of Bcl-2 and Bcl-x_L is less well documented.[36,37] Co-regulation might explain a positive correlation between expression of all three Bcl-2 proteins.[38,39] In contrast, protein activity showed a negative correlation of Bcl-2 and Bcl-x_L activity, which could either indicate a suppression of Bcl-2 activity by highly active Bcl-x_L or an induction of Bcl-x_L activity when Bcl-2 activity is low.[40]

To validate the accuracy of in silico protein activity estimation and to detect cell type specific as well as subcellular localization IHC and immunoblot on (i)CCA tissue and cell lines was performed. Thereby, we observed overexpression of Bcl-x_L and Mcl-1, but not Bcl-2, in iCCA compared with cholangiocytes. Given the described pattern of antiapoptotic Bcl-2 proteins in CCA, we assessed the efficacy of specific Bcl-2 protein inhibitors in vitro. Targeting specific Bcl-2 proteins with BH3-mimetics has proven potential in preclinical models of various solid tumours.[17,41,42] Our data pointed towards a hierarchy among Bcl-2 proteins in CCA. In all CCA cell lines investigated, response rates to the Bcl-x_L specific inhibitor Wehi-539 were higher compared with Mcl-1 and Bcl-2 inhibition. Furthermore, Bcl-x_L and Mcl-1 inhibition markedly sensitized the CCA cell lines to chemotherapeutic agents 5-Fluorouracil, Cisplatin, and Gemcitabine. A dependency of Cisplatin sensitivity on Bcl-x_L expression has been demonstrated in the context of farnesoid X receptor (FXR) agonists in CCA.[43] These results highlight the importance of Bcl-2 proteins and identify a dominant role of Bcl-x_L, and partly Mcl-1, as factors for therapy resistance in CCA paving the way to further translation.[44,45]

Exploiting the potential of our approach further, we demonstrate that combined inhibition of Mcl-1 and Bcl-x_L caused synergistic cell death increase that exceeded the effect of the chemotherapeutic approach. There are various preclinical studies demonstrating a dependency on the two proteins (Mcl-1 and Bcl-x_L) in cancer cells due to functional redundancy favouring a dual blockade.[9,46,47] The combination of Wehi-539 and S63845 is of great interest since it would be a treatment regimen that possibly does not require the use of chemotherapeutics. But there are still potential severe toxicities, such as platelet depletion, anaemia, and neutropenia since both Mcl-1 and Bcl-x_L are crucial for development of haematopoietic stem cells.[15,48–50] Intriguingly, recent research has shown that the combined inhibition of Mcl-1 and Bcl-x_L had an extremely potent, but yet tolerable effect on melanoma cells in vivo, suggesting that this combination could be a promising option.[51] However, further experiments involving in vivo models should be conducted in order to validate our in vitro approach, investigate potential toxicities and explore a possible translational potential.

Our data show that Bcl-x_L is of prognostic importance in CCA, since high expression identifies patients with better survival. Data on a predictive potential of Bcl-x_L in other tumour types is conflicting.[52–54]

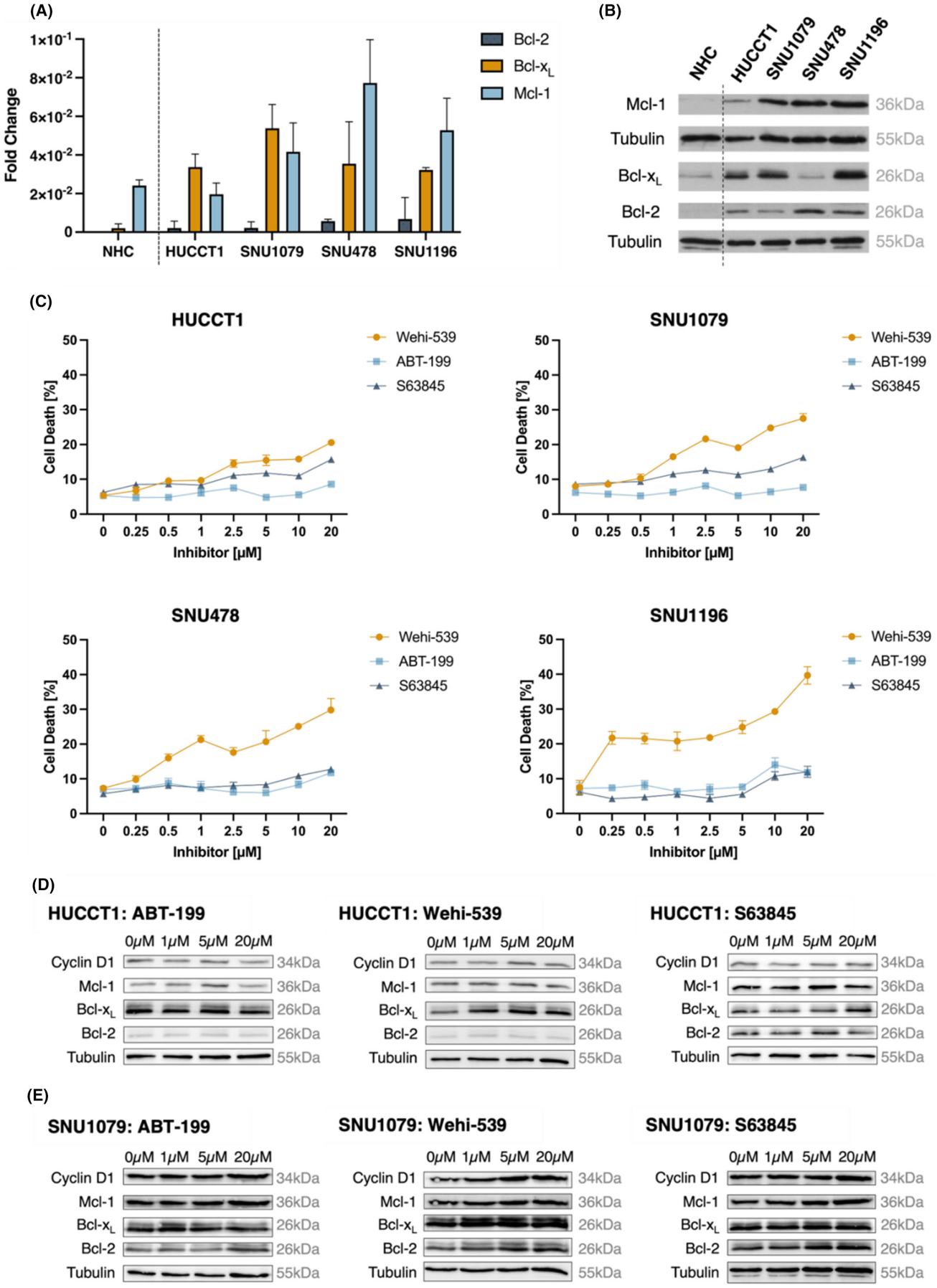


FIGURE 3 CCA cell lines overexpress antiapoptotic proteins and are sensitive to Bcl-x_L specific inhibitor Wehi-539. (A) RNA was extracted from non-transformed human cholangiocytes (NHC), human iCCA cells (HUCCT1, SNU1079) and human eCCA cells (SNU1196, SNU478) and cDNA was synthesized by reverse transcription. GAPDH was used as reference. Data is shown as relative quantification (Target Cp/GAPDH Cp), representing mean ± SD of three independent replicates (Bcl-x_L: HUCCT1 vs. NHC $p = .0024$; SNU1079 vs. NHC $p = .0305$; SNU478 vs. NHC p = ns; SNU1196 vs. NHC $p = .0034$; Mcl-1: HUCCT1 vs. NHC $p = .9836$; SNU1079 vs. NHC $p = .0921$; SNU478 vs. NHC $p = .0031$; SNU1196 vs. NHC $p = .3361$; Bcl-2: HUCCT1 vs. NHC $p = .9746$; SNU1079 vs. NHC $p = .4212$; SNU478 vs. NHC $p = .5653$; SNU1196 vs. NHC $p = 0.9701$). One-way ANOVA with the post hoc Dunnett's multiple comparison test was used to compare the results of CCA cell lines with those of NHC. (B) The levels of anti-apoptotic proteins Bcl-2, Bcl-x_L, and Mcl-1 were analyzed in non-transformed cholangiocytes (NHC) and the employed CCA cell lines (HUCCT1, SNU1079, SNU478, SNU1196) by western immunoblotting. Tubulin served as an internal control. (C) Four CCA cell lines (HUCCT1, SNU1079, SNU478, SNU1196) were plated in 24 well plates and treated with seven different concentrations (0.25, 0.5, 1, 2.5, 5, 10, and 20 μM) of specific inhibitors for Bcl-2 (ABT-199), Bcl-x_L (Wehi-539) and Mcl-1 (S63845) for 48 h. Cell death was measured by flow cytometry. Data represent mean ± SD of three independent replicates. (D, E) The employed CCA cell lines were plated in 12 well plates and treated with three different concentrations (1, 5, and 20 μM) of highly specific small Bcl-2 (ABT-199), Bcl-x_L (Wehi-539) and Mcl-1 (S63845) inhibitors for 48 h. Bcl-2, Bcl-x_L, Mcl-1, and Cyclin D1 were analyzed by immunoblotting. Tubulin served as an internal control

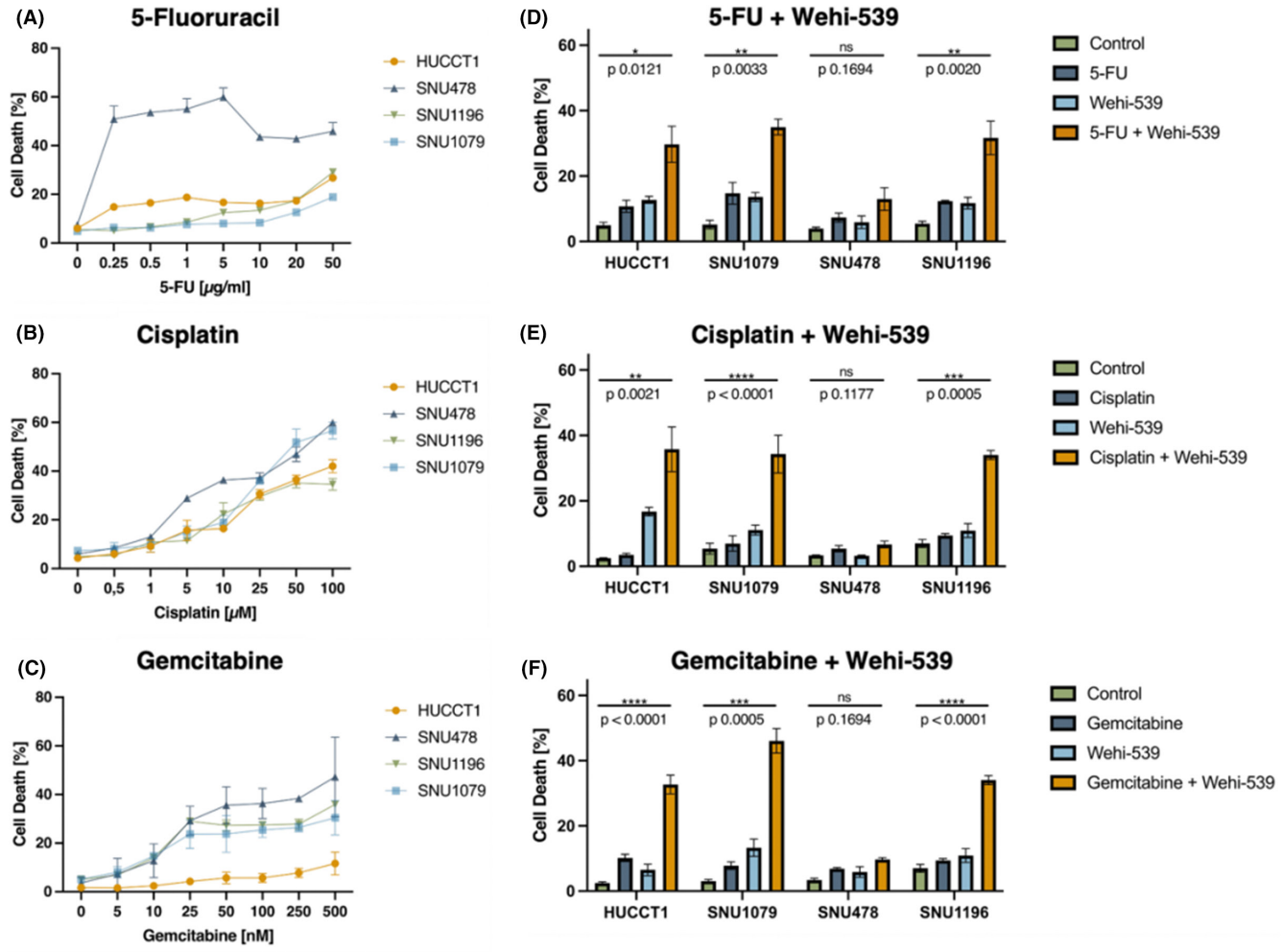


FIGURE 4 Treatment with Wehi 539 sensitizes CCA cell lines towards chemotherapy. (A–C) Four CCA cell lines (HUCCT1, SNU1079, SNU478, SNU1196) were seeded in 24 well plates and treated with seven different concentrations of three chemotherapeutic agents (5-Fluoruracil, Gemcitabine, and Cisplatin) for 48 h. Cell Death analysis was performed using flow cytometry. Data represent mean ± SD of three independent replicates. (D–F) Four CCA cell lines were seeded in 12 well plates and treated with the employed chemotherapeutic agents (5-Fluoruracil (D), Cisplatin (E) or Gemcitabine (F)), with the specific Bcl-x_L small molecule inhibitor Wehi-539 as well as a combination of each chemotherapeutic agent and Wehi-539 for 48 h. The used concentrations were individually adapted to the cell lines. (HUCCT1: Wehi-539 2.5 μM, 5-FU 0.25 μg/mL, Cisplatin 2 μM, Gemcitabine 250 nM; SNU1079: Wehi-539 1.25 μM, 5-FU 5 μg/mL, Cisplatin 5 μM, Gemcitabine 10 nM; SNU478: Wehi-539 5 μM, 5-FU 0.01 μg/mL, Cisplatin 1 μM, Gemcitabine 5 nM; SNU1196: Wehi-539 5 μM, 5-FU 20 μg/mL, Cisplatin 5 μM, Gemcitabine 10 nM). Cell Death analysis was measured using flow cytometry showing a synergistic effect of both treatments on cell death. Data represent mean ± SD of three independent replicates. Synergism of treatment effects was statistically evaluated using a two-way ANOVA test followed by a post hoc Dunnett T3 multiple comparison test

Among others, in colorectal cancer, Bcl-x_L expression indicates poor prognosis.[53,55] In NSCLC, Bcl-x_L fails to inform prognosis.[56] For childhood leukaemia and pancreatic ductal adenocarcinoma, high Bcl-x_L indicates longer survival.[57,58] In the context of CCA, employing large CCA cohorts uncovered that anatomical subtype is key: high level of Bcl-x_L on both, RNA and protein level, is associated with a favourable course, especially when only considering pCCA. Distinct molecular and biological features of iCCA, pCCA, and dCCA have been described regarding morphological, clinical, as well as molecular aspects.[59,60] In our study, the subgroups were similarly sized and did not show any great differences between the clinicopathological data (Table S5). However, the subgroups showed a different correlation with Bcl-x_L expression, highlighting the importance to establish specific clinical studies as well as treatment regimen for each anatomical subgroup. Differentiation between "small" and "large" bile duct iCCA revealed no significant difference concerning survival or Bcl-x_L expression, even though a tendency towards better survival within the small duct subgroup was observed, which is consistent with recent research.[61] Pathologically, large bile duct iCCA shows high similarities with pCCA and dCCA.[62]

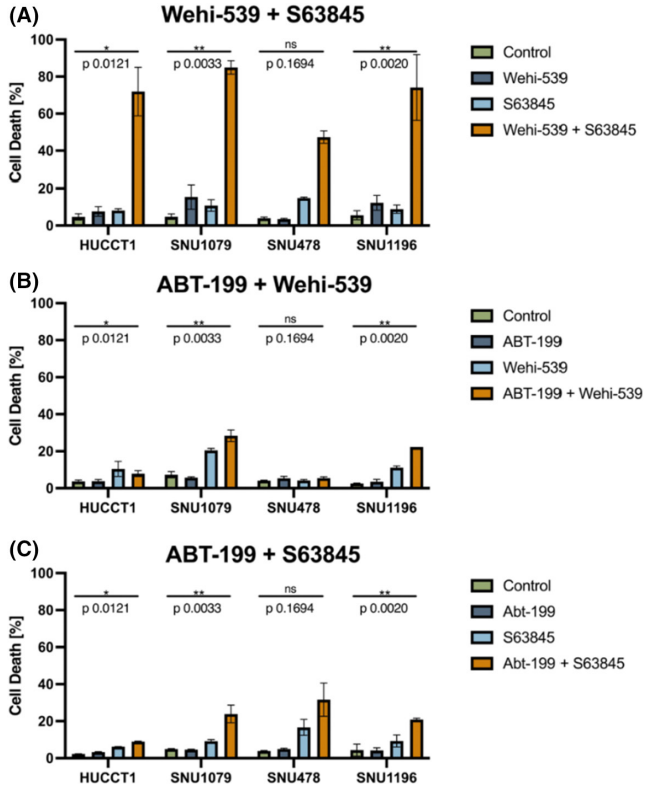


FIGURE 5 Combined treatment with Wehi-539 and S63845 leads to highly synergistic cell death increase. (A–C) Four CCA cell lines were seeded in 12 well plates and treated with a specific Bcl-x_L inhibitor (Wehi-539) and Mcl-1 inhibitor (S63845) (A) or with a specific Bcl-2 inhibitor (ABT-199) and Bcl-x_L inhibitor (Wehi-539) (B) or a Mcl-1 inhibitor (S63845) (C) as well as with a combination of both for 48 h. The used concentrations were individually adapted to each cell line (HUCCT1: Wehi-539 2.5 μM, S63845 10 μM, ABT-199 20 μM; SNU1079: Wehi-539 1.25 μM, S63845 10 μM, ABT-199 20 μM; SNU478: Wehi-539 5 μM, S63845 10 μM, ABT-199 20 μM; SNU1196: Wehi-539 5 μM, S63845 10 μM, ABT-199 20 μM). Cell Death analysis was measured using flow cytometry showing a synergistic effect of both treatments on cell death. Data represent mean ± SD of three independent replicates. Synergism of treatment effects was statistically evaluated using a two-way ANOVA test followed by a post hoc Dunnett T3 multiple comparison test

Recently approved novel therapeutic options, targeting altered genes (i.e., FGFR2) in iCCA actually may not be effective in eCCA, because of the differing mutational pattern and the lack of druggable FGFR alterations in this subtype - making specific therapeutic approaches even more indispensable.[62–67] There is sparse data available investigating distinguishing features of distinct CCA subtypes in the context of a deregulated cell death. Thus, our study adds significant knowledge with translational potential and yet highlights the biological uniqueness of specific CCA subtypes.[68] Reaching beyond prognostic impact, Bcl-x_L may have a predictive value in CCA, since its expression identifies patients with a favourable outcome during chemotherapy.

In conclusion, our study provides preclinical evidence for Bcl-x_L as a promising target for CCA treatment. We proved in vitro that the inhibition of Bcl-x_L and Mcl-1 augments the efficacy of common chemotherapeutics and induces synergistic cell death. Furthermore, our results indicate that Bcl-x_L is a prognostic factor in CCA when considering the anatomical subtype of the tumour.

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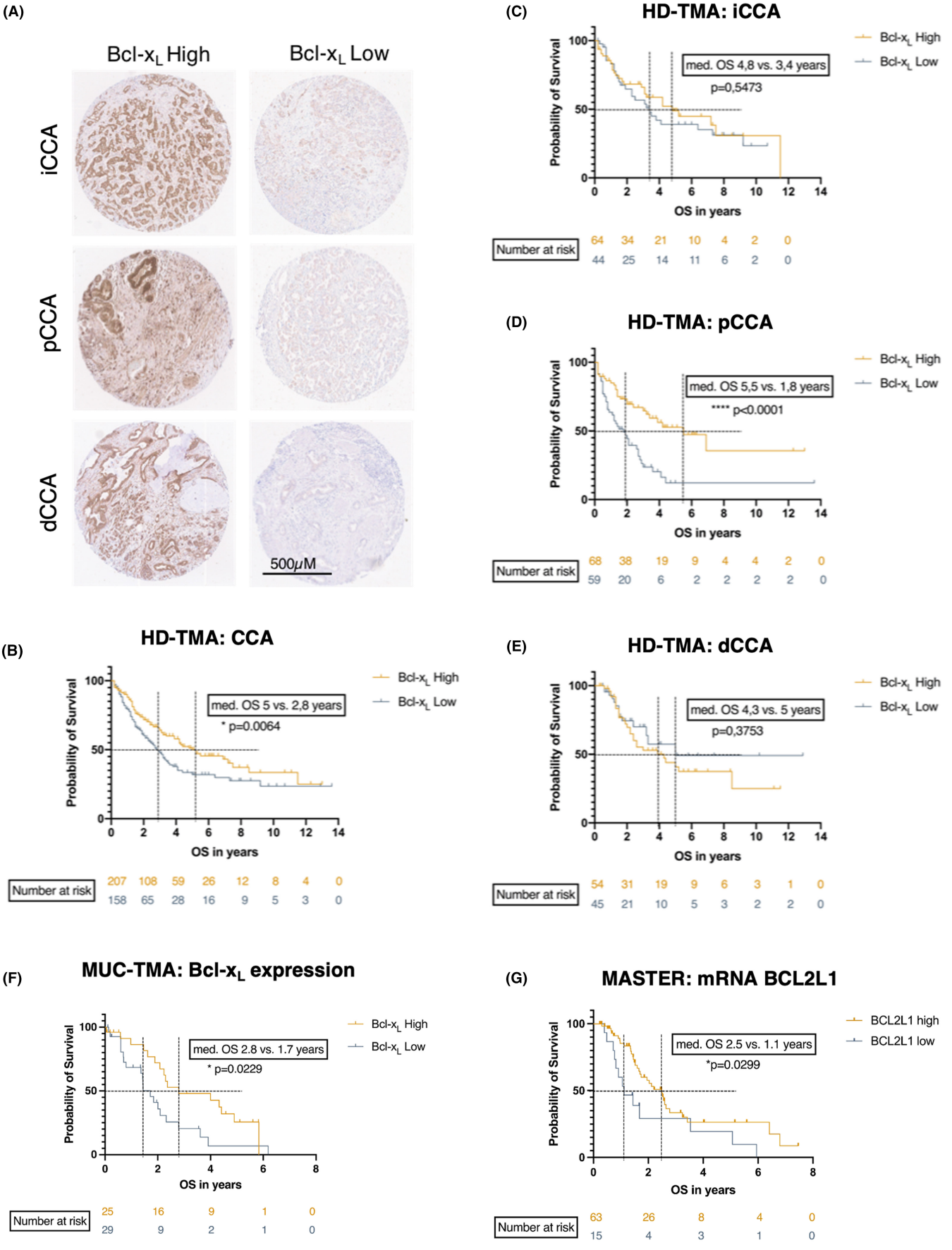
FIGURE 6 Different impact of Bcl-x_L expression in pCCA versus iCCA and dCCA. (A) Immunohistochemical staining for Bcl-x_L was performed on a Tissue Micro Array containing iCCA, pCCA, and dCCA spots. Representative images are shown. (B–E) Kaplan-Meyer survival analysis showing the correlation between Bcl-x_L expression and survival within the HD-TMA cohort. (B) all included CCA patients: Significant longer OS in patients with high Bcl-x_L expression (5 vs. 2.8 years, p = .0064). (C, E) iCCA/dCCA: no significant impact of Bcl-x_L expression on outcome (4.8 vs 3.4 years, $p = .5473$; 4.3 vs. 5 years, $p = .3753$ respectively) (D) pCCA: High Bcl-x_L expression is related with longer OS survival (5.5 vs. 1.8 years, $p < .0001$). (F) Kaplan–Meyer survival analysis showing the correlation between high Bcl-x_L expression that correlates with longer patient survival within the Munich TMA cohort (2.8 vs. 1.7 years, $p = .0229$) (G) Kaplan–Meyer survival analysis of the DKTK/MASTER cohort showing a significant longer survival of patients with high BCL2L1 tpm values (2.5 vs. 1.3 years, $p = .042$)



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CONFLICTS OF INTERESTS

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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