Frontiers in Bioscience Landmark

Front. Biosci. (Landmark Ed) **2023**; 28(4): 75
https://doi.org/10.31083/j.fbl2804075

Original Research

Prognostic Significance of DNA Topoisomerase II Alpha (TOP2A) in Cholangiocarcinoma

Khaa Hoo Ong[1,2,3], Hong-Yue Lai[4], Ding-Ping Sun[1], Tzu-Ju Chen[2,5], Steven Kuan-Hua Huang[6,7], Yu-Feng Tian[8], Chia-Lin Chou[2,8], Yow-Ling Shiue[3,9], Ti-Chun Chan[10,11], Chien-Feng Li[9,10,11,12], Yu-Hsuan Kuo[3,13,14,*]

[1]Division of Gastroenterology & General Surgery, Department of Surgery, Chi Mei Medical Center, 710 Tainan, Taiwan
[2]Department of Medical Technology, Chung Hwa University of Medical Technology, 717 Tainan, Taiwan
[3]Institute of Biomedical Sciences, National Sun Yat-sen University, 804 Kaohsiung, Taiwan
[4]Department of Pharmacology, School of Medicine, China Medical University, 404333 Taichung, Taiwan
[5]Department of Clinical Pathology, Chi Mei Medical Center, 710 Tainan, Taiwan
[6]Division of Urology, Department of Surgery, Chi Mei Medical Center, 710 Tainan, Taiwan
[7]Department of Medical Science Industries, College of Health Sciences, Chang Jung Christian University, 711 Tainan, Taiwan
[8]Division of Colon and Rectal Surgery, Department of Surgery, Chi Mei Medical Center, 710 Tainan, Taiwan
[9]Institute of Precision Medicine, National Sun Yat-sen University, 804 Kaohsiung, Taiwan
[10]Department of Medical Research, Chi Mei Medical Center, 710 Tainan, Taiwan
[11]National Institute of Cancer Research, National Health Research Institutes, 704 Tainan, Taiwan
[12]Trans-Omic Laboratory for Precision Medicine, Chi Mei Medical Center, 710 Tainan, Taiwan
[13]Division of Hematology and Oncology, Department of Internal Medicine, Chi-Mei Medical Center, 71004 Tainan, Taiwan
[14]College of Pharmacy and Science, Chia Nan University, 71710 Tainan, Taiwan
*Correspondence: beethovan@gmail.com (Yu-Hsuan Kuo)
Academic Editor: Elisa Belluzzi
Submitted: 24 December 2022 Revised: 3 March 2023 Accepted: 27 March 2023 Published: 19 April 2023

Abstract

Background: Cholangiocarcinoma (CCA) is a malignant tumor with an increasing incidence worldwide. Although radiation therapy has improved the therapeutic efficiency of CCA treatment, differential expression of genes among cholangiocarcinoma subtypes has been revealed through precise sequencing. However, no specific molecular therapeutic targets or biomarkers have been figured out for use in precision medicine, and the exact mechanism by which antitumorigenic effects occur is still unclear. Therefore, it is necessary to conduct further studies on the development and mechanisms associated with CCA. **Methods**: We examined the clinical data and pathological features of patients with cholangiocarcinomas. We investigated the associations between DNA Topoisomerase II Alpha (TOP2A) expression and patient outcomes, such as metastasis-free survival (MFS) and disease-specific survival (DSS), as well as clinical characteristics and pathological results. **Results**: *TOP2A* expression was shown to be upregulated in CCA tissue sections by immunohistochemistry staining and data mining. Moreover, we observed that the *TOP2A* expression correlated with clinical features, such as the primary tumor stage, histological variants, and patients with hepatitis. Furthermore, high expression of *TOP2A* was associated with worse survival outcomes in terms of the overall survival ($p < 0.0001$), disease-specific survival ($p < 0.0001$), and metastasis-free survival ($p < 0.0001$) compared with patients in the low *TOP2A* expression group. This indicates that a high level of *TOP2A* expression is related to an unfavorable prognosis. **Conclusions**: Our results show that *TOP2A* is highly expressed in CCA tissues, and its upregulation is correlated with the primary disease stage and poor prognosis significantly. Consequently, *TOP2A* is a prognostic biomarker and a novel therapeutic target for the treatment of CCA.

Keywords: cholangiocarcinoma; DNA topoisomerase (ATP-hydrolyzing activity) activity; topoisomerase II α (TOP2A); prognostic biomarker

1. Introduction

Cholangiocarcinoma, which is a type of cancer that affects the epithelial cells, can arise from various locations of the bile duct [1]. Depending on its location, cholangiocarcinoma can be classified as intrahepatic, perihilar, or distal. Intrahepatic cholangiocarcinoma starts from the secondary bile ducts located close to the liver, while perihilar cholangiocarcinoma occurs between the cystic duct and the secondary bile ducts. Distal cholangiocarcinoma is found between the ampulla and the origin of the cystic duct [2,3]. According to previous studies, the risk factors for cholangiocarcinoma (CCA) are sporadic and are correlated with geographic variations [4,5]. For example, infection with hepatobiliary flukes is associated with chronic inflammation and is also correlated with the development of CCA in individuals from Southeast Asia [6]; Hepatolithiasis is also a risk factor of developing CCA (mainly intrahepatic cholangiocarcioma (iCCA)) in Asians [5]. In the West, pri-

mary sclerosing cholangitis (PSC) carries the greatest risk for patients with CCA [7,8]. Accordingly, CCA is diagnosed within two years of developing PSC. Although risk factors for CCA development, such as smoking and alcohol, have been identified in PSC patients, direct research data are lacking [9].

The *TOP2A* gene is responsible for regulating the chromosome bond pathway by encoding DNA topoisomerase, which in turn controls the topological state of DNA transcription and replication [10]. Studies have linked the expression of *TOP2A* with various levels of cancer progression and the development of nasopharyngeal carcinoma [11], breast cancer [12], adrenal cancer [13], and endometrial cancer [14]. Abnormal expression of *TOP2A* is often linked to irregular cell proliferation, while decreased expression of the gene can lead to changes in several molecular signaling pathways, such as the β-catenin pathway in pancreatic cancer and EPK/AKT in colon cancer [15–17]. Furthermore, *TOP2A* is considered a prognostic factor or a driver gene that affects the survival of patients with different types of cancer [16].

In recent years, few studies involving genetic factors (tumor suppressors and oncogenes) and epigenetic alterations associated with CCA progression have been conducted [18]. However, there is no definitive result to identify the major oncogenes or suppressor genes associated with CCA development. In this study, we explore the molecular role of *TOP2A* in CCA and try to identify the biological functions of *TOP2A* in CCA progression.

2. Material and Methods

2.1 Analysis of Published Transcriptomic Datasets

The gene expression profile of cholangiocarcinoma (GSE26566) was investigated through the Gene Expression Omnibus (GEO) database. Significant gene expression changes were identified, particularly those in molecular pathways involving DNA topoisomerase (ATP-hydrolyzing activity) activity in Gene Ontology (GO:2000371), through comparative and functional analyses. The databases were analyzed, and focus was placed on p-values of <0.01 and log2-transformed expression fold changes of >0.1.

2.2 Cholangiocarcinoma Patient Tissues

The Chi Mei Medical Center enrolled 182 iCCA patients who underwent curative surgery between 1990 and 2010. Patients with metastatic disease or nodal metastases were excluded. This study was authorized by the Institutional Review Board (IRB) of The Chi Mei Medical Center with approval number 09912003. All participants provided their informed consent. Patients' demographics and clinical details was collected retrospectively, including pathological characteristics, oncological survival follow-up, and cause of death. The Tumor, Node, Metastasis (TNM) system created by the eighth edition American Joint Committee on Cancer (AJCC) in 2017 was used to measure the tumor stage. Two pathologists examined the tumor samples.

2.3 Immunohistochemistry (IHC) Staining

The formalin-fixed tissues were embedded in paraffin and sectioned into 10 μm sections. Subsequently, immunohistochemical (IHC) staining of *TOP2A* was conducted using a primary monoclonal antibody directed towards *TOP2A* (1:200, EP1102Y, Epitomics, Cambridge, UK) for a duration of 1 hour. To detect the primary antibodies, the ChemMate EnVision kit (DAKO, K5001, Carpinteria, CA, USA) was utilized. The secondary antibody was incubated with the slides for 30 minutes and then developed with 3,3-diaminobenzidine for 5 minutes. Subsequently, the slides were counterstained with hematoxylin. Cholangiocarcinoma expressing *TOP2A* was utilized as a positive control, while rabbit serum IgG replaced the primary antibody as a negative control. Two pathologists used the formula H-score = SPi (i + 1) to compute the H-score, where Pi stands for the percentage of stained tumor cells in different intensities ranging from 0% to 100% and i for the degree of staining (0 to 3+). The two pathologists evaluated the slides concurrently and decided on an H-score in case there were any scoring disagreements. Based on the median H-score, the immunostaining was categorized as having low or high expression levels.

2.4 Real-Time Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

The relative expression and knockdown efficacy of the *TOP2A* gene in iCCA cell lines were determined using RT-PCR, as previously published [19]. Total RNA was collected from iCCA cell lines, and real-time RT-PCR was employed to determine the transcription level of TOP2A. The mRNA abundance of *TOP2A* (Hs01032137_mL) was assessed using predesigned TaqMan assay reagents (Cat. No. Hs01032137_mL, Applied Biosystems, Waltham, MA, USA) and the ABI StepOnePlusTM system (Applied Biosystems, Waltham, MA, USA), with POLR2A (Hs01108291_mL) used as the internal control for normalization.

2.5 Cell Culture

The SNU1079 and SNU1196 cell lines were procured from a cell bank based in Seoul, South Korea. Initially, these cell lines were cultured in ACL-4 medium supplemented with 5% heat-inactivated fetal bovine serum. After establishment, the cell cultures were maintained in RPMI 1640 medium and supplemented with 10% heat-inactivated fetal bovine serum. These cells were cultured under controlled conditions in a humidified incubator at 37 °C in the presence of 5% CO_2 and 95% air as previously described [20]. On the other hand, the Huh28 and HuCCT1 cell lines were obtained from a cell bank in Osaka, Japan. These cell lines were cultured in either Roswell Park Memorial Institute 1640 medium (HuCCT1) or minimal essential medium

(Huh28), which were both supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum as previously reported [21]. All the cell lines were authenticated by short tandem repeat genotyping (ThermoFisher, Waltham, MA, USA), periodically confirmed to be mycoplasma free using PlasmoTest (Invivogen, San Diego, CA, USA).

2.6 RNA Interference

The SNU1196 and Huh28 cell lines were modified to create stable TOP2A-silenced clones using lentiviral vectors pLKO.1-sh*LacZ* (TRCN0000072223: 5′-TGTTCGCATTATCCGAACCAT-3′) and pLKO.1-sh*TOP2A* (#1: TRCN0000049280: 5′-GCTCCAAATCAA TATGTGATT-3′; #2: TRCN0000049278: 5′-GCCCAA GTGTTCTTTAGCTTT-3′), which were obtained from the Taiwan National RNAi Core Facility in Taipei, Taiwan. These cell lines initially had high *TOP2A* expression that was reduced by shRNAs against *TOP2A* (sh*TOP2A*). To generate viruses for the modification, HEK293 cells were transfected with the three vectors mentioned above, using Lipofectamine 2000 from Thermo Fisher Scientific in Waltham, MA, USA, as previously described [19].

2.7 Western Blotting

Primary antibodies against *TOP2A* (clone AA6, 1:500; Millipore, Beverly, MA, USA) were utilized, and a previously reported western blotting technique was utilized to assess *TOP2A* expression and the effect of *TOP2A* knockdown in SNU1196 and Huh28 cell lines. Glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used as a control for protein loading (6C5, 1:10,000; Millipore, Beverly, MA, USA) [22]. To immobilize the protein, cell lysates containing 25 μg of protein were separated on a 4% to 12% gradient NuPAGE gel (Invitrogen, Carlsbad, CA, USA) and transferred to polyvinylidene difluoride membranes (Amersham Biosciences, Buckinghamshire, UK). To prevent non-specific binding, membranes were blocked with TBST buffer and 5% skimmed milk at room temperature for one hour. Primary antibodies were then exposed to the membranes at 4 °C overnight. After that, the membranes were incubated with the secondary antibody for 1.5 hours at room temperature, and proteins were identified using a chemiluminescence system (Amersham Biosciences, Buckinghamshire, UK).

2.8 Bromodeoxyuridine (BrdU) Assay to Assess DNA Synthesis

The enzyme-linked immunosorbent assay (ELISA)-based and colorimetric bromodeoxyuridine (BrdU) assay (Roche Holding AG, Basel, Switzerland) was utilized to quantify DNA synthesis. At 24, 48, and 72 hours, the amount of DNA synthesis was measured in the *TOP2A*-knockdown or shLacA control SNU1196 and Huh28 cell lines. After three hours of BrdU incubation at 37 °C and 5% CO_2, the labeling medium was removed and the cells were fixed before being incubated with an anti-BrdU-POD solution. Using an ELISA reader (Promega Corp., Madison, WI, USA), the absorbance at 450 nm was measured, with the reference absorbance set to 690 nm.

2.9 Migration and Invasion Assays

The experimental protocol for cell migration and invasion was carried out in accordance with a previously documented procedure [22]. For the cell invasion experiment, the 24-well Collagen-based Cell Invasion Assay from Millipore, Beverly, MA, USA, and Falcon HTS FluoroBlok 24-well inserts from BD Biosciences, Franklin Lakes, NJ, USA, were used. The inserts were rehydrated using serum-free medium and then placed in the upper chamber, which contained a serum-free suspension with an equal number of cells. Over a 12- to 24-hour incubation period, the cells were allowed to migrate towards the lower chamber, which contained a medium with 10% fetal bovine serum. Following removal of the non-invading cells from the upper chamber, the invading cells were stained, lysed in extraction buffer, and then transferred to 96-well plates for 560 nm colorimetric readings.

2.10 Functional Annotation

In order to determine the unknown functions of *TOP2A* in iCCA, the transcription level of *TOP2A* and its coexpressed genes contained in the cholangiocarcinoma dataset (n = 51, Firehose Legacy, TCGA) were analyzed to establish correlations. Subsequently, the top 200 differentially expressed genes with positive or negative correlations with *TOP2A* were selected for functional annotation using the Gene Ontology (GO) classification system and rated by fold enrichment.

2.11 Statistical Analysis

The statistical analyses were conducted using SPSS software (version 28, IBM Corp., Chicago, IL, USA). The Chi-square test was utilized to evaluate the association between *TOP2A* expression stage and clinicopathologic characteristics. Local recurrence-free survival (LRFS), metastasis-free survival (MFS), and disease-specific survival (DSS) were calculated from the beginning of therapy to the date of the event, and the latest follow-up date was noted for patients who were lost to follow-up during the study period. Kaplan-Meier analysis was employed to generate survival curves, and log-rank tests were utilized to identify prognostic differences between groups. All analyses were performed using two-sided significance tests, and a *p*-value less than 0.05 was considered significant.

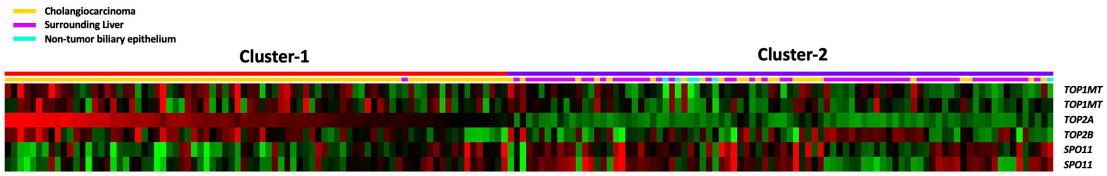

Fig. 1. Data mining of genes expression compared between cholangiocarcinoma, surrounding liver and non-tumor biliary epithelium from the public domain (GSE26566).

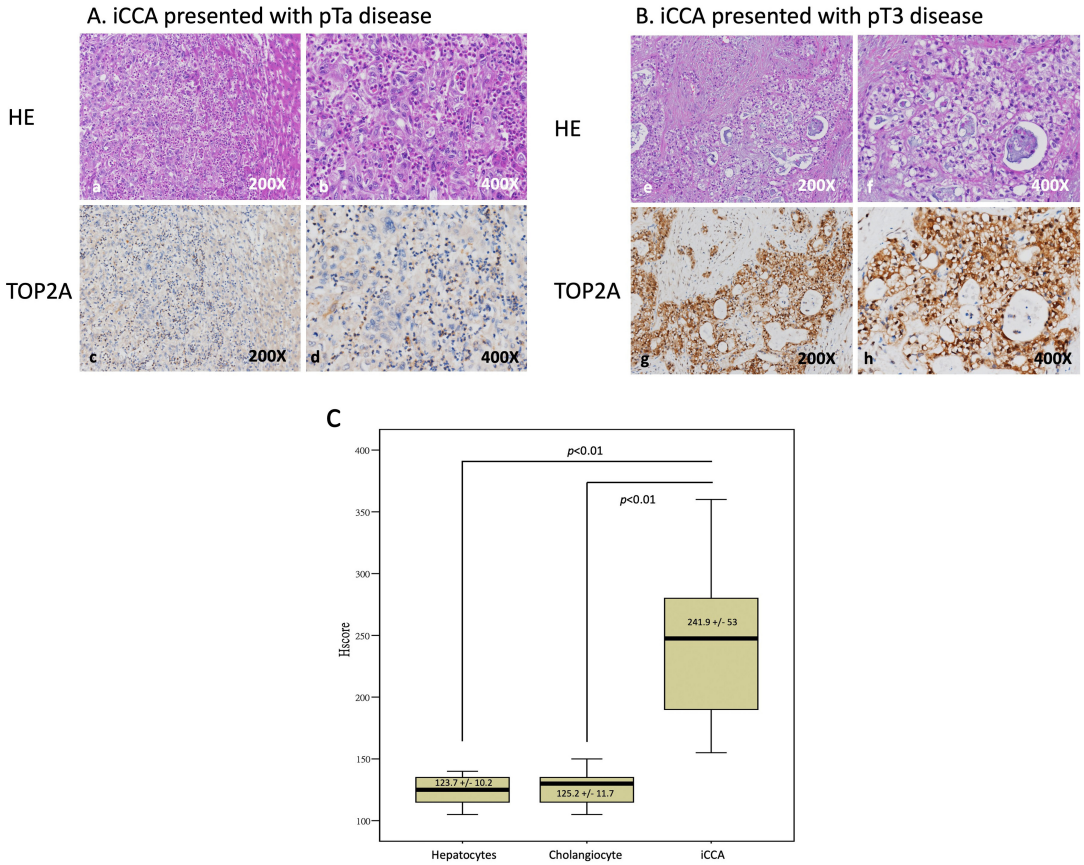

Fig. 2. Identify the *TOP2A* expression in iCCA tissues. (A) In low-stage tumor tissues, *TOP2A* positive cells are weak detected (a–d). (B) In high-stage tumor tissues, tumor cells showed positive *TOP2A* staining (e–h). (C) A comparison of H-score showed significantly higher *TOP2A* expression in iCCA cells than hepatocytes and cholangiocytes (N = 182 in each group).

3. Results

3.1 TOP2A Expression was Associated with DNA Topoisomerase Activity in CCA

We mined the public CCA transcriptome dataset (GSE26566) and compared the results with DNA topoisomerase (ATP-hydrolyzing activity) activity (GO:2000371). We discovered that five genes were significantly associated with DNA topoisomerase (ATP-hydrolyzing activity) activity (Table 1), and *TOP2A* was found to be a high-ranking differently expressed candidate gene that showed a significant difference ($p < 0.0001$) and a log-ratio of >0.1 (Table 1). Moreover, the heat map data mining results also showed that expression of DNA topoisomerase-related genes differed between CCA tissue and the surrounding liver and non-tumor biliary epithelium tissues, and *TOP2A* was shown to be highly expressed in CCA patients (Fig. 1). The above data demonstrate that *TOP2A* might play a critical role in cancer progression in CCA patients.

3.2 The Association between TOP2A Expression and Important Clinic Pathological Parameters in CCA

Our previous results indicate that high expression of *TOP2A* might be correlated with CCA progression. We further analyzed associations between *TOP2A* expression and the clinicopathologic characteristics of CCA patients. We included data from 182 iCCA patients, 108 males and 74 females, where 107 patients were older than 65 and 75 patients were younger than 65. These cases were classified as 105 of the large duct type and 77 of the small duct type based on histological variants; 61 cases were classified as

Table 1. Summary of the alterations of gene associated with DNA topoisomerase (ATP-hydrolyzing activity) activity (GO:2000371) in cholangiocarcinoma (GSE26566).

Probe	CCA vs Non-tumor[#]		CCA vs Normal intrahepatic bile duct[&]		Gene Symbol	Molecular function	Biological process
	log ratio	*p*-value	log ratio	*p*-value			
ILMN_1686097	1.7717	<0.0001*	1.7928	<0.0001*	*TOP2A*	DNA topoisomerase (ATP-hydrolyzing) activity, protein homodimerization activity, histone deacetylase binding, protein heterodimerization activity, ATP binding, chromatin binding, DNA-dependent ATPase activity, nucleotide binding, protein C-terminus binding, ubiquitin binding, protein kinase C binding, drug binding	DNA ligation, DNA topological change, positive regulation of apoptosis, phosphoinositide-mediated signaling, DNA repair, chromosome segregation, DNA replication, apoptotic chromosome condensation
ILMN_1777663	0.3019	0.0018*	0.339	0.1852	*TOP2B*	DNA topoisomerase (ATP-hydrolyzing) activity, histone deacetylase binding, protein heterodimerization activity, ATP binding, chromatin binding, nucleotide binding, protein C-terminus binding, protein kinase C binding	DNA topological change
ILMN_1659651	0.2556	<0.0001*	0.6474	<0.0001*	*TOP1MT*	DNA topoisomerase (ATP-hydrolyzing) activity, DNA topoisomerase type I activity	DNA topological change
ILMN_1796508	0.185	0.0010*	0.4342	0.0122*			
ILMN_1735572	0.0875	0.1265	0.1408	0.3608	*TOP1*	DNA topoisomerase (ATP-hydrolyzing) activity, chromatin binding, DNA topoisomerase type I activity, protein binding	DNA topological change
ILMN_1687970	–0.1256	0.0006*	–0.1417	0.1639	*SPO11*	DNA topoisomerase (ATP-hydrolyzing) activity, hydrolase activity, DNA binding, ATP binding	DNA topological change, female gamete generation, spermatogenesis, meiotic recombination, meiosis
ILMN_1796655	–0.1309	0.0009*	–0.0693	0.5052			DNA topological change, female gamete generation, spermatogenesis, meiotic recombination, meiosis

#, Comparing cholangiocarcinoma (CCA, n = 104) to surrounding liver (n = 59) and normal intrahepatic bile duct (n = 6); &, Comparing cholangiocarcinoma (CCA, n = 104) to normal intrahepatic bile duct (n = 6); * statistically significant.

Table 2. Correlations between *TOP2A* expression and other important clinicopathological parameters in primary localized IHCC.

Parameter	Category	Case No.	*TOP2A* expression		*p*-value
			Low	High	
Gender	Male	108	51	57	0.365
	Female	74	40	34	
Age (years)	<65	107	49	58	0.175
	≥65	75	42	33	
Hepatitis	Hepatitis B	72	38	34	0.002*
	Hepatitis C	29	22	7	
	Non-B, non-C	81	31	50	
Intrahepatic lithiasis	Not identified	102	56	46	0.135
	Present	80	35	45	
Surgical margin	R0	163	84	79	0.225
	R1	19	7	12	
Primary tumor (T)	T1	87	56	21	0.001*
	T2	61	24	37	
	T3	34	11	23	
Histological variants	Large duct type	105	42	63	0.002*
	Small duct type	77	49	28	
Histological grade	Well differentiated	61	31	30	0.887
	Moderately differentiated	66	34	32	
	Poorly differentiated	55	26	29	

* Statistically significant.

well-differentiated, 66 as moderately differentiated, and 55 as poorly differentiated according to histological grades. A total of 163 cases were classified as R0 and 19 cases were classified as R1 based on the surgical margin, and in terms of the primary tumor size, 87 cases were classified as T1, 61 as T2, and 34 as T3 (Table 2). Taken together, our results indicate that *TOP2A* overexpression is highly correlated with the co-occurrence of hepatitis (p-value = 0.002), the primary tumor size (p-value = 0.001), and the histological variants (p-value = 0.002) of clinicopathological parameters in cholangiocarcinoma patients with iCCA (Table 2). Moreover, we determined that *TOP2A* is overexpressed in cholangiocarcinoma patients (Fig. 1). We confirmed this by IHC staining of cholangiocarcinoma patient tissue sections (Fig. 2). Moreover, we calculated the immunohistochemical staining H-score which showed significantly higher in iCCA cells than hepatocytes and cholangiocytes (Fig. 3). These data suggest that *TOP2A* expression is significantly associated with clinicopathological variables in iCCA patients.

3.3 Identified TOP2A in Comparison with the Patient Survival Analysis

To determine whether *TOP2A* expression is correlated with CCA patients' survival outcomes, we analyzed the association between *TOP2A* expression and the overall survival rate, disease-specific survival rate, local recurrence-free survival rate, and metastasis-free survival rate in CCA patients. For overall survival, an R0 stage surgical margin, T1 stage primary tumor, and low *TOP2A* expression (Fig. 3A–C) were associated with better outcomes in both the univariate and multivariate analyses (Table 3). The disease-specific survival endpoints analysis showed the same results whereby R0 stage, T1 stage, and low *TOP2A* expression patients had better outcomes (Table 3) (Fig. 3D–F) than other patients. Moreover, patients with a T1 stage primary tumor, large duct type histological variant, and histological low grade were shown to have better outcomes in the univariate analysis of local recurrence-free survival (Table 4). Importantly, an R0 stage surgical margin, T1 stage primary tumor, and low *TOP2A* expression were all significantly associated with better outcomes (Fig. 3G–I) in both the univariate analysis and the multivariate analysis of local recurrence-free survival and metastasis-free survival (Table 4) (Fig. 3J–L).

Taken together, our results indicate that *TOP2A* is highly expressed in CCA patients, and high *TOP2A* expression is correlated with poor prognosis and notably worse overall survival ($p = 0.0011$, Fig. 3C), disease-specific survival ($p < 0.0001$, Fig. 3F), local recurrence-free survival ($p < 0.0001$, Fig. 3I), and metastasis-free survival ($p < 0.0001$, Fig. 3L) rates.

3.4 Bioinformatic Analysis and Functional Prediction of TOP2A

To determine the unknown functions of *TOP2A* in IHCC, a set of the top 200 differentially expressed genes with positive (**Supplementary Table 1**) or negative corre-

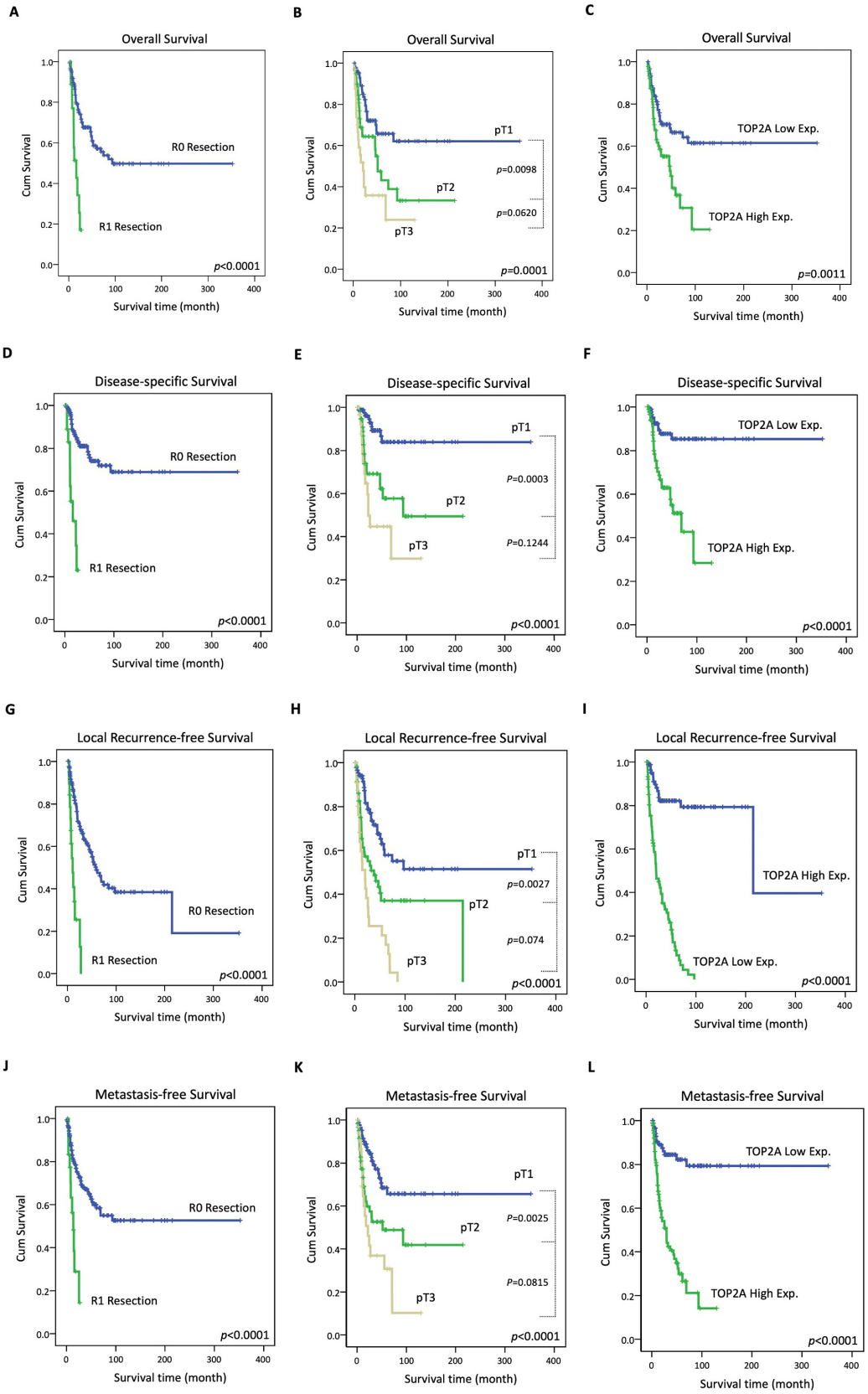

Fig. 3. Kaplan–Meier survival curves in the CCA patients according to surgical margin, primary tumor staging and *TOP2A* expression. R0 Resection, pT1 stage and low *TOP2A* expression correlated with better overall survival (A,B,C), better disease-specific survival (D,E,F), better local recurrence-free survival (G,H,I) and better metastasis-free survival (J,K,L).

Table 3. Univariate log-rank and multivariate analyses for overall and disease-specific survivals in primary localized IHCC.

Parameter	Category	Case No.	Overall survival					Disease-specific survival				
			Univariate analysis		Multivariate analysis			Univariate analysis		Multivariate analysis		
			No. of event	*p*-value	R.R.	95% CI	*p*-value	No. of event	*p*-value	R.R.	95% CI	*p*-value
Gender	Male	108	50	0.0254*	1	-	0.087	9	0.0072*	1	-	0.044*
	Female	74	21		1.569	0.937–2.629	-	32		2.158	1.021–4.558	-
Age (years)	<65	107	37	0.2626	-	-	-	28	0.2125	-	-	-
	≥65	75	34		-	-	-	13		-	-	-
Hepatitis	Hepatitis B	72	32	0.2379	-	-	-	16	0.4561	-	-	-
	Hepatitis C	29	8		-	-	-	19		-	-	-
	Non-B, non-C	81	31		-	-	-	6		-	-	-
Intrahepatic lithiasis	Not identified	102	36	0.2831	-	-	-	19	0.1613	-	-	-
	Present	80	35		-	-	-	22		-	-	-
Surgical margin	R0	163	59	<0.0001*	1	-	0.002*	31	<0.0001*	1	-	<0.001*
	R1	19	12		2.913	1.466–5.789		10		4.962	2.196–11.213	
Primary tumor (T)	T1	87	25	0.0001*	1	-	0.037*	9	<0.0001*	1	-	0.020*
	T2	61	27		1.608	0.920–2.812	-	19		2.769	1.234–6.211	-
	T3	34	19		2.317	1.205–4.455	-	13		3.281	1.321–8.153	-
Histological variants	Large duct type	105	43	0.4281	-	-	-	27	0.1984	-	-	-
	Small duct type	77	28		-	-	-	14		-	-	-
Histological grade (Differentiation)	Well	61	20	0.1663	-	-	-	12	0.3881	-	-	-
	Moderately	66	28		-	-	-	16		-	-	-
	Poorly	55	23		-	-	-	13		-	-	-
TOP2A Exp.	Low expression	91	28	0.0011	1	-	0.036*-	10	<0.0001*	1	-	0.002*-
	High expression	91	43		1.714	1.035–2.838	-	31		1.929	1.198–3.886	-

* Statistically significant.

Table 4. Univariate log-rank and multivariate analyses for local recurrence-free and metastasis-free survivals in primary localized IHCC.

Parameter	Category	Case No.	Local recurrence-free survival					Metastasis-free survival				
			Univariate analysis		Multivariate analysis			Univariate analysis		Multivariate analysis		
			No. of event	*p*-value	R.R.	95% CI	*p*-value	No. of event	p-value	R.R.	95% CI	*p*-value
Gender	Male	108	54	0.2170	-	-	-	21	0.1008	-	-	-
	Female	74	31		-	-	-	44		-	-	-
Age (years)	<65	107	55	0.2993	-	-	-	42	0.2936	-	-	-
	≥65	75	30		-	-	-	23		-	-	-
Hepatitis	Hepatitis B	72	33	0.7333	-	-	-	26	0.8762	-	-	-
	Hepatitis C	29	13		-	-	-	11		-	-	-
	Non-B, non-C	81	39		-	-	-	28		-	-	-
Intrahepatic lithiasis	Not identified	102	41	0.0551	-	-	-	31	0.1000	-	-	-
	Present	80	44		-	-	-	34		-	-	-
Surgical margin	R0	163	71	<0.0001*	1	-	0.010*	54	<0.0001*	1		0.009*
	R1	19	14		2.702	1/294–5.640		11		2.674	1.306–5.474	
Primary tumor (T)	T1	87	28	<0.0001*	1	-	0.051	21	<0.0001*	1	-	0.046*
	T2	61	32		1.741	0.961–3.154		26		1.759	0.978–3.164	
	T3	34	25		1.899	0.958–3.764		18		1.912	0.965–3.788	
Histological variants	Large duct type	105	58	0.0085*	1	-	0.988	43	0.0759	-	-	-
	Small duct type	77	27		1.108	0.592–1.751		22		-	-	-
Histological grade (Differentiation)	Well	61	28	0.0299*	1	-	0.829	22	0.1794	-	-	-
	Moderately	66	27		1.154	0.630–2.114		22		-	-	-
	Poorly	55	30		1.208	0.638–2.283		21		-	-	-
TOP2A Exp.	Low expression	91	15	<0.0001*	1	-	<0.001	14	<0.0001*	1	-	<0.001
	High expression	91	70		4.658	2.481–8.745		51		4.695	2.514–8.768	

* Statistically significant.

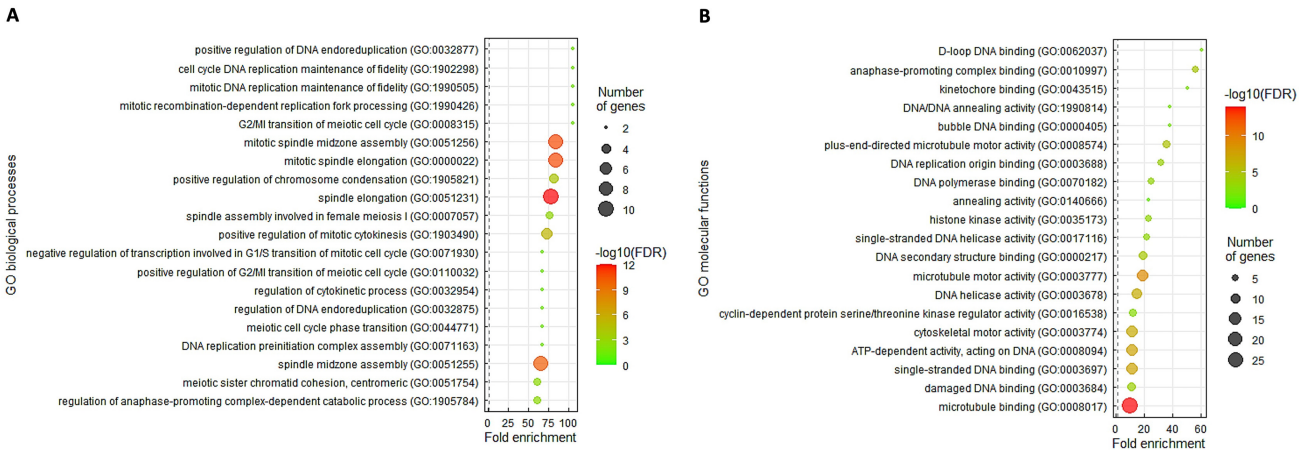

Fig. 4. The characteristic GO terms enriched in *TOP2A* upregulation. The top 200 differentially expressed genes with positive relationship to *TOP2A* were annotated utilizing the GO classification system depending on (A) biological processes and (B) molecular functions and were rated by fold enrichment.

lations (**Supplementary Table 2**) with *TOP2A* were downloaded from the cholangiocarcinoma dataset (n = 51, Firehose Legacy, TCGA). These genes were then utilized for a functional annotation analysis using the Gene Ontology (GO) classification system. The biological processes most positively correlated with *TOP2A* were positive regulation of DNA endoreduplication (GO: 0032877, fold enrichment: >100), cell cycle DNA replication maintenance of fidelity (GO: 1902298, fold enrichment: >100), and mitotic DNA replication maintenance of fidelity (GO: 1990505, fold enrichment: >100) (Fig. 4A). DNA repair protein *RAD51* homolog 1 (*RAD51*) and breast cancer type 2 susceptibility protein (*BRCA2*) genes, involved in most of the biological processes mentioned above, were also identified. As for molecular functions, the most positively correlated term with *TOP2A* was D-loop DNA binding (GO: 0062037, fold enrichment: 60.85) (Fig. 4B), and the *RAD51*-associated protein 1 (*RAD51AP1*) gene was identified.

3.5 TOP2A Promotes the Cell Proliferation, Migration, and Invasion of CCA Cell Lines

In order to assess the impact of *TOP2A*, we initially measured the levels of endogenous *TOP2A* expression in four different cholangiocarcinoma cell lines. We found that SNU1196 and Huh28 cells had the highest amounts of *TOP2A* transcripts and protein expression (Fig. 5A). We then used short hairpin RNA (shRNA) to successfully knock down *TOP2A* in both SNU1196 (Fig. 5B, left) and Huh28 (Fig. 5B, right) cell lines. *TOP2A*-silenced SNU1196 (Fig. 5E, left) and Huh28 (Fig. 5E, right) cells had considerably reduced proliferation (viability). We also looked at *TOP2A*'s role in cholangiocarcinoma cell migration and invasion. *TOP2A* knockdown significantly reduced SNU1196 (Fig. 5C, left and Fig. 5D, left) and Huh28 (Fig. 5C, right and Fig. 5D, right) cell migratory and invasive abilities.

4. Discussion

Cholangiocarcinoma is a heterogeneous group of tumors that initiate from a number of cells from the biliary tree [23]. According to previous research, the risk and the molecular mechanisms associated with CCA pathogenesis involve inflammation and cholestasis or CCA development [24]. Accordingly, it has been observed that CCA patients have increased total serum bilirubin, alkaline phosphatase, and gamma-glutamyl transpeptidase levels or obstructive jaundice [25,26]. Although no specific biomarkers for CCA have been identified, some references indicate that Carbohydrate antigen 19-9 (CA 19-9) could be used as be a marker for the detection of CCA, since CA 19-9 levels >100 U/mL have been reported to be a sensitive method for diagnosing CCA patients [27]. However, markers such as CA 19-9 can also be detected in gastric, pancreatic, colorectal, and gynecologic cancer patients or in individuals with other diseases, such as cholestasis, liver injury, and benign biliary obstruction [27–29]. Although researchers have explored the risks [30–32] and therapeutic methods [33–35] associated with CCA and have explored imaging diagnosis [36], there is no direct biomarker or powerful therapeutic target available to improve the treatment efficiency.

TOP2A is a member of the TOP2 family that plays a vital role in promoting transcriptional initiation in DNA replication, chromosome condensation, and mitosis [37]. Furthermore, *TOP2A* has been reported to play a crucial role in cancer progression. For example, *TOP2A* expression is usually increased or down-regulated depending on the presence of Erb-B2 Receptor Tyrosine Kinase 2 (ERBB2). Based on this characteristic, these two genes are often co-tested before treating patients with Herceptin as an anticancer drug [38,39]. Other studies have demonstrated that *TOP2A* is upregulated in lung cancer patients by IHC staining, and it has also been discovered that *TOP2A* expression is correlated with poor overall survival in lung can-

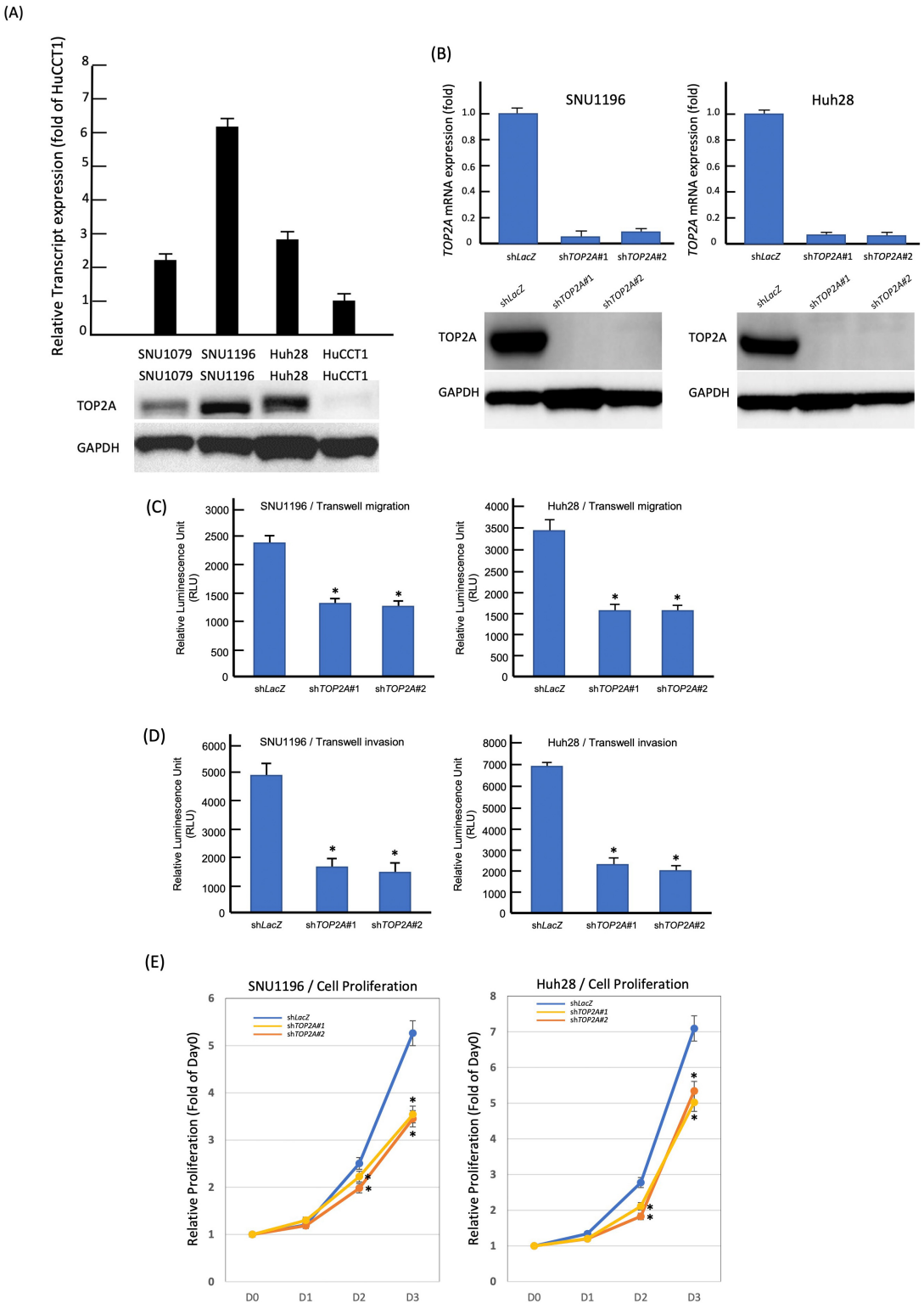

Fig. 5. *TOP2A* expression promotes the growth of CCA cells *in vitro*. (A) SNU1196 and Huh28 cells had the highest amounts of *TOP2A* mRNA and protein expression among four CCA cell lines. (B) The two cell lines with high endogenous *TOP2A* expression were stably silenced by a lentiviral vector bearing one of the two clones of *TOP2A* shRNA with different sequences for both SNU1196 (left panel) and Huh28 (right panel) cells. (C) Cell migration ability was significantly reduced in *TOP2A*- knockdown SNU1196 and Huh28 cell lines. (D) Cell invasion ability was significantly reduced in *TOP2A*- knockdown SNU1196 and Huh28 cell lines. (E) Using an ELISA-based colorimetric assay to assess the rate of BrdU uptake, cell proliferation was significantly reduced in *TOP2A*-knockdown SNU1196 and Huh28 cell lines (* $p < 0.05$, ** $p < 0.01$).

cer patients [40]. Moreover, high expression of *TOP2A* and EZH2 is positively correlated with aggressive disease progression in prostate cancer patients [41]. Recently, *TOP2A* was considered as a therapeutic target of anti-cancer drugs. For example, *TOP2A* may serve as a predictor of responses to anthracycline therapy in breast cancer patients, and etoposide is used for the treatment of different cancers (lung cancer, ovarian, lymphoma, and acute myeloid leukemia) [42–44]. Furthermore, several researchers have clarified that topoisomerase II inhibitors affect cancer cell proliferation by inducing apoptosis, altering metabolism, and regulating the JAK2-STAT1-CXCL1 molecular pathway [45,46]. In addition, topoisomerase inhibitors directly affect nucleic acid metabolism, which hints at their potential lethality [47]. Nevertheless, the functions and regulation of *TOP2A* in CCA patients were still unknown prior to this study.

In order to gain insight into the impact of *TOP2A* on CCA progression and prognosis, we conducted an analysis of *TOP2A* expression in patient tissues using an online database. The results revealed that *TOP2A* expression was markedly elevated in CCA patients, particularly in those with advanced primary T stage and those with poor overall survival rates and R1 stage surgical margins.

DNA double-strand breaks (DSBs) can be repaired by homologous recombination (HR) or non-homologous end joining (NHEJ) in response to DNA damage [48]. Homologous recombination is a DNA repair process that involves *RAD51* recombinase and its regulator *BRCA2* [49]. Since topoisomerase II may also generate transient DSBs in DNA [50], it is not surprisingly that the *BRCA2*, *RAD51*, and *RAD51AP1* genes were found to be significantly positively correlated with *TOP2A* (**Supplementary Table 1** and Fig. 4). *BRCA2* is generally regarded as a tumor suppressor, and its mutation may contribute to an increased risk for the development of various cancers, especially breast and ovarian cancers [51]. However, the recovery of *BRCA2* function owing to secondary *BRCA2* mutation has been considered a mechanism associated with acquired resistance to cisplatin, suggesting that this genetic reversion is beneficial for cell survival [52]. In addition, high *RAD51* expression has also been indicated to increase drug resistance and genome instability in tumor cells [53]. Moreover, as an *RAD51* activator, *RAD51AP1* upregulation has been correlated with inferior survival in hepatocellular carcinoma patients [54]. Collectively, the involvement of *TOP2A*, *BRCA2*, *RAD51*, and *RAD51AP1* in IHCC development deserves further investigation.

5. Conclusions

In summary, the expression of *TOP2A* has been recognized to be upregulated in CCA patients, and this was confirmed by immunohistochemistry staining of CCA tissue sections. The expression of *TOP2A* was significantly correlated with the primary tumor stage (according to AJCC stages) and histological variant (large duct type). Moreover, high expression of *TOP2A* is predictive of worse overall survival, disease-specific survival, and metastasis-free survival rates. In addition, increased expression of *TOP2A* may contribute to tumor progression in CCA patients. This information indicates that *TOP2A* can be considered for use in future prospective prognostic analyses. Moreover, we need more evidence to clarify the molecular mechanisms and explore the biological functions of *TOP2A* to determine its potential as a therapeutic target for CCA in clinical trials.

Availability of Data and Materials

The transcriptome dataset (GSE26566) used in this study is publicly available in the Gene Expression Omnibus (GEO) database, which is maintained by the National Center for Biotechnology Information in Bethesda, MD, USA.

Author Contributions

Conceptualization—KHO and YHK; methodology—KHO, HYL, DPS, TJC, SKHH, YFT, CLC, YLS, TCC, and CFL; investigation—KHO, HYL, DPS, TJC, SKHH, YFT, CLC, YLS, TCC, and CFL; formal analysis—KHO, HYL, DPS, TJC, SKHH, YFT, CLC, YLS, TCC, and CFL; resources—CLC, YLS, TCC, and CFL; validation—KHO, HYL, DPS, TJC, SKHH, and YFT; visualization—KHO, HYL, DPS, TJC, SKHH, and YFT; writing - original draft—KHO and YHK; writing - review & editing—KHO and YHK; funding acquisition—YHK; supervision—KHO and YHK. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

All participants in this study provided informed consent before their samples were deposited in the biobank. The Ethics Committee and Institutional Review Board of Chi Mei Medical Center (IRB09912003) approved the study's use of deidentified tumor samples from the biobank, indicating that the research was conducted in compliance with ethical guidelines outlined in the Declaration of Helsinki and regulations set forth by the government.

Acknowledgment

Not applicable.

Funding

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2804075.

References

[1] Razumilava N, Gores GJ. Cholangiocarcinoma. Lancet. 2014; 383: 2168–2179.

[2] Battal M, Gürbulak B, Bostanci O, Yılmaz MB, Ozdenkaya Y, Karatepe O. Cholangiocarcinoma presenting with hypercalcemia and thrombocytopenia. Case Reports in Medicine. 2014; 2014: 246817.

[3] Razumilava N, Gores GJ. Combination of gemcitabine and cisplatin for biliary tract cancer: a platform to build on. Journal of Hepatology. 2011; 54: 577–578.

[4] Khan SA, Davidson BR, Goldin RD, Heaton N, Karani J, Pereira SP, *et al*. Guidelines for the diagnosis and treatment of cholangiocarcinoma: an update. Gut. 2012; 61: 1657–1669.

[5] Tyson GL, El-Serag HB. Risk factors for cholangiocarcinoma. Hepatology. 2011; 54: 173–184.

[6] Shin HR, Oh JK, Lim MK, Shin A, Kong HJ, Jung KW, *et al*. Descriptive epidemiology of cholangiocarcinoma and clonorchiasis in Korea. Journal of Korean Medical Science. 2010; 25: 1011–1016.

[7] Matchar DB, Samsa GP, Matthews JR, Ancukiewicz M, Parmigiani G, Hasselblad V, *et al*. The Stroke Prevention Policy Model: linking evidence and clinical decisions. Annals of Internal Medicine. 1997; 127: 704–711.

[8] Razumilava N, Gores GJ. Classification, diagnosis, and management of cholangiocarcinoma. Clinical Gastroenterology and Hepatology: the Official Clinical Practice Journal of the American Gastroenterological Association. 2013; 11: 13–21.e1.

[9] Wadsworth CA, Lim A, Taylor-Robinson SD, Khan SA. The risk factors and diagnosis of cholangiocarcinoma. Hepatology International. 2013; 7: 377–393.

[10] Lee SK, Wang W. Roles of Topoisomerases in Heterochromatin, Aging, and Diseases. Genes. 2019; 10: 884.

[11] Lan J, Huang HY, Lee SW, Chen TJ, Tai HC, Hsu HP, *et al*. TOP2A overexpression as a poor prognostic factor in patients with nasopharyngeal carcinoma. Tumour Biology. 2014; 35: 179–187.

[12] Zhu L, Ma N, Wang B, Zhou C, Yan Y, Wang K, *et al*. Clinical analysis of 21-gene recurrence score test in hormone receptor-positive early-stage breast cancer. Oncology Letters. 2019; 17: 5469–5480.

[13] Xiao H, Xu D, Chen P, Zeng G, Wang X, Zhang X. Identification of Five Genes as a Potential Biomarker for Predicting Progress and Prognosis in Adrenocortical Carcinoma. Journal of Cancer. 2018; 9: 4484–4495.

[14] Liu L, Lin J, He H. Identification of Potential Crucial Genes Associated With the Pathogenesis and Prognosis of Endometrial Cancer. Frontiers in Genetics. 2019; 10: 373.

[15] Chen T, Sun Y, Ji P, Kopetz S, Zhang W. Topoisomerase IIα in chromosome instability and personalized cancer therapy. Oncogene. 2015; 34: 4019–4031.

[16] Pei YF, Yin XM, Liu XQ. TOP2A induces malignant character of pancreatic cancer through activating β-catenin signaling pathway. Biochimica et Biophysica Acta. Molecular Basis of Disease. 2018; 1864: 197–207.

[17] Zhang R, Xu J, Zhao J, Bai JH. Proliferation and invasion of colon cancer cells are suppressed by knockdown of TOP2A. Journal of Cellular Biochemistry. 2018; 119: 7256–7263.

[18] Andersen JB, Thorgeirsson SS. Genetic profiling of intrahepatic cholangiocarcinoma. Current Opinion in Gastroenterology. 2012; 28: 266–272.

[19] Chan TC, Hsing CH, Shiue YL, Huang SK, Hsieh KL, Kuo YH, *et al*. Angiogenesis Driven by the CEBPD-hsa-miR-429-VEGFA Signaling Axis Promotes Urothelial Carcinoma Progression. Cells. 2022; 11: 638.

[20] Ku JL, Yoon KA, Kim IJ, Kim WH, Jang JY, Suh KS, *et al*. Establishment and characterisation of six human biliary tract cancer cell lines. British Journal of Cancer. 2002; 87: 187–193.

[21] Merdrignac A, Angenard G, Allain C, Petitjean K, Bergeat D, Bellaud P, *et al*. A novel transforming growth factor beta-induced long noncoding RNA promotes an inflammatory microenvironment in human intrahepatic cholangiocarcinoma. Hepatology Communications. 2018; 2: 254–269.

[22] Chan TC, Chen YT, Tan KT, Wu CL, Wu WJ, Li WM, *et al*. Biological significance of MYC and CEBPD coamplification in urothelial carcinoma: Multilayered genomic, transcriptional and posttranscriptional positive feedback loops enhance oncogenic glycolysis. Clinical and Translational Medicine. 2021; 11: e674.

[23] Rimassa L, Personeni N, Aghemo A, Lleo A. The immune milieu of cholangiocarcinoma: From molecular pathogenesis to precision medicine. Journal of Autoimmunity. 2019; 100: 17–26.

[24] Labib PL, Goodchild G, Pereira SP. Molecular Pathogenesis of Cholangiocarcinoma. BMC Cancer. 2019; 19: 185.

[25] Nakeeb A, Lipsett PA, Lillemoe KD, Fox-Talbot MK, Coleman J, Cameron JL, *et al*. Biliary carcinoembryonic antigen levels are a marker for cholangiocarcinoma. American Journal of Surgery. 1996; 171: 147–153.

[26] Nehls O, Gregor M, Klump B. Serum and bile markers for cholangiocarcinoma. Seminars in Liver Disease. 2004; 24: 139–154.

[27] Levy C, Lymp J, Angulo P, Gores GJ, Larusso N, Lindor KD. The value of serum CA 19-9 in predicting cholangiocarcinomas in patients with primary sclerosing cholangitis. Digestive Diseases and Sciences. 2005; 50: 1734–1740.

[28] Koopmann J, Thuluvath PJ, Zahurak ML, Kristiansen TZ, Pandey A, Schulick R, *et al*. Mac-2-binding protein is a diagnostic marker for biliary tract carcinoma. Cancer. 2004; 101: 1609–1615.

[29] Patel AH, Harnois DM, Klee GG, LaRusso NF, Gores GJ. The utility of CA 19-9 in the diagnoses of cholangiocarcinoma in patients without primary sclerosing cholangitis. The American Journal of Gastroenterology. 2000; 95: 204–207.

[30] Shaib Y, El-Serag HB. The epidemiology of cholangiocarcinoma. Seminars in Liver Disease. 2004; 24: 115–125.

[31] Bergquist A, Glaumann H, Persson B, Broomé U. Risk factors and clinical presentation of hepatobiliary carcinoma in patients with primary sclerosing cholangitis: a case-control study. Hepatology. 1998; 27: 311–316.

[32] Scott J, Shousha S, Thomas HC, Sherlock S. Bile duct carcinoma: a late complication of congenital hepatic fibrosis. Case report and review of literature. The American Journal of Gastroenterology. 1980; 73: 113–119.

[33] Khan SA, Davidson BR, Goldin R, Pereira SP, Rosenberg WMC, Taylor-Robinson SD, *et al*. Guidelines for the diagnosis and treatment of cholangiocarcinoma: consensus document. Gut. 2002; 51: VI1–9.

[34] Jarnagin WR, Shoup M. Surgical management of cholangiocarcinoma. Seminars in Liver Disease. 2004; 24: 189–199.

[35] Ortner MA. Photodynamic therapy in cholangiocarcinomas. Best Practice & Research. Clinical Gastroenterology. 2004; 18: 147–154.

[36] Nieveen van Dijkum EJ, de Wit LT, Gouma DJ. Imaging of the hepatobiliary tract. The New England Journal of Medicine. 1997; 337: 1391–1392.

[37] Madabhushi R. The Roles of DNA Topoisomerase IIβ in Transcription. International Journal of Molecular Sciences. 2018; 19: 1917.

[38] Chen JR, Chien HP, Chen KS, Hwang CC, Chen HY, Yeh KY, *et al*. Amplification of HER2 and TOP2A and deletion of TOP2A genes in a series of Taiwanese breast cancer. Medicine. 2017; 96: e5582.

[39] Huijsmans CJJ, van den Brule AJC, Rigter H, Poodt J, van

der Linden JC, Savelkoul PHM, *et al*. Allelic imbalance at the HER2/TOP2A locus in breast cancer. Diagnostic Pathology. 2015; 10: 56.

[40] Ma W, Wang B, Zhang Y, Wang Z, Niu D, Chen S, *et al*. Prognostic significance of TOP2A in non-small cell lung cancer revealed by bioinformatic analysis. Cancer Cell International. 2019; 19: 239.

[41] Labbé DP, Sweeney CJ, Brown M, Galbo P, Rosario S, Wadosky KM, *et al*. TOP2A and EZH2 Provide Early Detection of an Aggressive Prostate Cancer Subgroup. Clinical Cancer Research: an Official Journal of the American Association for Cancer Research. 2017; 23: 7072–7083.

[42] Wang S, Zimmermann S, Parikh K, Mansfield AS, Adjei AA. Current Diagnosis and Management of Small-Cell Lung Cancer. Mayo Clinic Proceedings. 2019; 94: 1599–1622.

[43] Wu CC, Li TK, Farh L, Lin LY, Lin TS, Yu YJ, *et al*. Structural basis of type II topoisomerase inhibition by the anticancer drug etoposide. Science. 2011; 333: 459–462.

[44] Hirsch FR, Scagliotti GV, Mulshine JL, Kwon R, Curran WJ, Jr, Wu YL, *et al*. Lung cancer: current therapies and new targeted treatments. Lancet. 2017; 389: 299–311.

[45] Wasim L, Chopra M. Synergistic anticancer effect of panobinostat and topoisomerase inhibitors through ROS generation and intrinsic apoptotic pathway induction in cervical cancer cells. Cellular Oncology. 2018; 41: 201–212.

[46] Du J, Liu S, He J, Liu X, Qu Y, Yan W, *et al*. MicroRNA-451 regulates stemness of side population cells via PI3K/Akt/mTOR signaling pathway in multiple myeloma. Oncotarget. 2015; 6: 14993–15007.

[47] Chowdhury SR, Majumder HK. DNA Topoisomerases in Unicellular Pathogens: Structure, Function, and Druggability. Trends in Biochemical Sciences. 2019; 44: 415–432.

[48] Vítor AC, Huertas P, Legube G, de Almeida SF. Studying DNA Double-Strand Break Repair: An Ever-Growing Toolbox. Frontiers in Molecular Biosciences. 2020; 7: 24.

[49] West SC. Molecular views of recombination proteins and their control. Nature Reviews. Molecular Cell Biology. 2003; 4: 435–445.

[50] Deweese JE, Osheroff N. The DNA cleavage reaction of topoisomerase II: wolf in sheep's clothing. Nucleic Acids Research. 2009; 37: 738–748.

[51] Moynahan ME. The cancer connection: BRCA1 and BRCA2 tumor suppression in mice and humans. Oncogene. 2002; 21: 8994–9007.

[52] Dhillon KK, Swisher EM, Taniguchi T. Secondary mutations of BRCA1/2 and drug resistance. Cancer Science. 2011; 102: 663–669.

[53] Klein HL. The consequences of Rad51 overexpression for normal and tumor cells. DNA Repair. 2008; 7: 686–693.

[54] Zhuang L, Zhang Y, Meng Z, Yang Z. Oncogenic Roles of RAD51AP1 in Tumor Tissues Related to Overall Survival and Disease-Free Survival in Hepatocellular Carcinoma. Cancer Control. 2020; 27: 1073274820977149.