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Original Article

27-Hydroxycholesterol in bile duct tissue promotes cholangiocarcinoma progression through estrogen receptor signaling

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Abstract: Background: The oxysterol 27-hydroxycholesterol (27-HC) is widely produced in human tissues, functions as a selective estrogen receptor modulator (SERM), is implicated in the progression of estrogen receptor (ER)-positive cancers. While 27-HC is abundant in bile, its SERM role in extrahepatic cholangiocarcinoma (eCCA) remains unclear. Therefore, this study aims to test the hypothesis that 27-HC promotes eCCA cell proliferation via ER activation. Methods: Oxysterol levels and the expression of ERs and proliferation-related genes were compared between eCCA tissues (N = 17) and noncancerous extrahepatic bile ducts (N = 6). The effects of 27-HC on cell proliferation were evaluated in two human cholangiocarcinoma cell lines: ERα-expressing intrahepatic CCA-1 and ERβ-expressing extrahepatic TFK-1 cells. Results: 27-HC and mRNA expression levels of ERα and ERβ were significantly higher in eCCA tissues than in noncancerous tissues. Expression levels of cMYC, HIF-1α, and VEGFα expression levels were elevated in eCCA tissues with high ERα and/or ERβ expression. In both cell lines, 27-HC (1-1,000 nM) dose-dependently enhanced cell proliferation for 48 h, similar to that of 17β-estradiol; these effects were blocked by ER inhibitors ICI-182,780 and PHTPP. Conclusions: These findings indicate that 27-HC promotes eCCA cell proliferation through ERα- and ERβ-mediated SERM-like effects, highlighting that 27-HC and ERs as potential therapeutic targets in eCCA.

Keywords: SERM, ER, CCA, 27-HC, extrahepatic bile duct

Introduction

Extrahepatic cholangiocarcinoma (eCCA) is a gastrointestinal malignancy associated with a poor prognosis. Surgical resection remains the only potentially curative treatment; however, complete resection is often difficult unless the disease is diagnosed early [1]. For patients with unresectable eCCA, radiation therapy and/or cytotoxic chemotherapy with combinations of gemcitabine, cisplatin, and S-1 have been used [2]. More recently, immune-targeted therapies have been introduced for eCCA treatment. Studies report clinical efficacy for gemcitabine and cisplatin (GC) combined with durvalumab, an anti-programmed death (PD)-ligand 1 antibody [3], as well as GC combined with pembrolizumab, an anti-PD-1 antibody [4]. In parallel, the molecular pathogenesis of eCCA and development of molecularly targeted therapies beyond immunotherapy are undergoing active investigation [5]. In the future, identifying specific gene expression profiles and protein markers in eCCA tissue obtained through biopsy or surgical resection may enable the selection of optimal, personalized treatment strategies for individual patients.

Estrogen is a sex steroid hormone essential to the female reproductive system and the nonreproductive systems of both sexes. It exerts its biological effects by binding to cytoplasmic estrogen receptors (ERα or ERβ), after which the hormone-receptor complex translocates to the nucleus. The actions of estrogen include its growth-promoting effect on ER-expressing nor-

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Table 1. The characteristics of the enrolled patients

Group	eCCA	NC
Number of patients [male:female]	11:6	3:1
Age (years)	73.2 ± 1.8	67.0 ± 7.6
Bilirubin (μmol/L)	1.44 ± 0.21*	0.86 ± 0.43
ALP (IU/L)	689.2 ± 121.0*	361.5 ± 249.4
γ-GPT (IU/L)	328.6 ± 78.7	215.8 ± 162.2
LAP (IU/L)	135.7 ± 20.1	104.0 ± 41.4
ALT (IU/L)	57.3 ± 12.3*	32.5 ± 25.4
AST (IU/L)	34.5 ± 3.9	25.0 ± 8.1
CA-19-9 (IU/mL)	317.7 ± 137.1	64.1 ± 282.6
CEA (ng/mL)	7.5 ± 3.9	3.8 ± 8.0
Cancer Stage [I:II:III]‡	4:7:5	

Data are shown as mean ± S.E. *$P<0.05$ shows significant difference to the NC group by unpaired Student's *t* test. ‡Cancer stage was diagnosed by the TNM staging system stands for tumor, node, and metastasis. Cancer stage in a patient of the eCCA group was not diagnosed by uncertain reason. Abbreviations: *ALP*, alkaline phosphatase; *ALT*, alanine aminotransferase; *AST*, aspartate aminotransferase; *CA-19-9*, carbohydrate antigen19-9; *CEA*, carcinoembryonic antigen; *eCCA*, extrahepatic cholangiocarcinoma; *γ-GPT*, γ-glutamyl transpeptidase; *LAP*, leucine aminopeptidase; *NC*, non-cancer; *M*, male; *F*, Female.

mal and neoplastic cells [6]. Studies report estrogen involvement in tumor progression in multiple malignancies, including breast [7], ovary [8], endometrium [9], prostate [10], thyroid [11], bladder [12], and lung cancers [13]. Intrahepatic cholangiocarcinoma (iCCA) is another malignancy in which estrogen stimulates tumor cell proliferation, invasion, and angiogenesis [14-16]. However, no studies have addressed ER expression in eCCA or its relationship to estrogen signaling and eCCA growth.

Estrogens, including 17β-estradiol (E2), are well-established ER agonists. Compounds such as tamoxifen, a widely used breast cancer therapy, exert tissue-specific agonistic or antagonistic effects on ERs. These compounds are collectively known as selective ER modulators (SERMs) [17]. This tissue specificity likely results from ligand-induced conformational changes in the 3-dimensional structure of ERs, which influence the recruitment of tissue-specific coregulatory proteins. 27-Hydroxycholesterol (27-HC) was the first endogenous ER ligand to exhibit SERM activity [18, 19]. This oxysterol is synthesized throughout the body from cholesterol by mitochondrial cytochrome P450 27A1 (CYP27A1) [20]. Hypercholesterolemia is a risk factor for ERα-positive breast cancer, and CYP27A1 expression in tumor cells and tumor-associated macrophages correlates with tumor grade in human breast cancer specimens [21]. Numerous studies report that 27-HC promotes breast cancer progression through ERα activation [22]. Beyond breast cancer, 27-HC promotes growth via ERα in endometrial cancer [23] and melanoma [24], and via ERβ in lung [25, 26] and prostate cancers [27].

eCCA predominantly occurs in older individuals and is diagnosed more frequently in men than in women [1]. Circulating estrogen levels do not differ between men and postmenopausal women; however, serum 27-HC concentrations tend to be higher in men [28, 29]. Bile also contains substantial amounts of 27-HC [30], and cholangiocytes may produce this oxysterol. Therefore, if eCCA cells express ERs, the abundant presence of 27-HC may stimulate their proliferation. This study aims to quantify 27-HC levels and ER expression in surgically resected eCCA samples and to investigate their associations with cholangiocarcinoma (CCA) cell proliferation.

Methods

Patients and sample collection

This study included 21 patients with suspected bile duct cancer resulting from biliary stenosis or hypertrophy who underwent pylorus-preserving pancreaticoduodenectomy at Tokyo Medical University Ibaraki Medical Center (Ibaraki, Japan) between June 2018 and February 2022 (**Table 1**). Common bile duct tissue samples were obtained from residual specimens following routine histopathological diagnosis by pathologists. Based on pathological findings, cases were classified as tubular adenocarcinoma (N = 17) or benign biliary disease (N = 4). Cancer stage was determined according to the TNM staging system, which includes tumor size, lymph node involvement, and distant metastasis. All specimens were immediately frozen and stored at -80°C until analysis.

Clinical and molecular parameters were compared between patients with eCCA (11 men

and six women) and those with benign biliary disease characterized based on inflammation or hyperplasia, who served as the non-cancer (NC) group (three men and one woman). For selected analyses, including gene expression and 27-HC levels in bile duct tissue, two normal regions of resected bile duct specimens were also included in the NC group.

Serum samples were collected from all patients before surgery and routine serological biochemistry analyses - including bilirubin, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), carbohydrate antigen19-9 (*CA-19-9)*, carcinoembryonic antigen (CEA), γ-glutamyl transpeptidase (γ-GPT), and dleucine aminopeptidase (LAP) - were performed. In 14 cases (11 eCCA and three benign diseases), bile was obtained intraoperatively from the common bile duct. Follow-up for up to 2,708 days was completed for 14 patients in the eCCA group and four patients in the NC group - until the last visit, death, or transfer to another hospital, whichever occurred first. All patients in the NC group survived the follow-up period.

The methods and purpose of the study were explained to patients, and informed consent was obtained before enrollment. The study protocol was approved by the Ethics Committee of Tokyo Medical University (Tokyo, Japan; IRB no. IB1810) and was conducted in accordance with the 1964 Helsinki Declaration.

Oxysterol analysis

Levels of twelve oxysterols - including 7α-hydroxycholesterol (7α-HC), 22R-hydroxycholesterol, 24S-hydroxycholesterol (24S-HC), 25-hydroxycholesterol (25-HC), 27-HC, 4β-hydroxycholesterol (4β-HC), cholesterol 5β,6β-epoxide (5β6β-EC), cholesterol 5α,6α-epoxide (5α6α-EC), 7-ketocholesterol (7-oxoC), 7β-hydroxycholesterol, 24(S),25-epoxycholesterol, and cholestane-3β,5α,6β-triol - were measured in serum, bile, and common bile duct tissue as previously described [31-33]. Briefly, after adding internal standards and butylated hydroxytoluene, serum (10 μL) and bile (20-100 μL) samples were saponified with 1 N ethanolic KOH at 37°C for 1 h, whereas bile duct tissue samples (5-50 mg) were saponified at 80°C for 20 min. Oxysterols were extracted with *n*-hexane, converted to picolinyl ester derivatives, and quantified using an HPLC-ESI-MS/MS system. During the assays, all samples were maintained under nitrogen to prevent autoxidation.

To clarify the association between 27-HC levels and mortality in patients with eCCA, 14 patients with eCCA who were followed up were stratified into two subgroups (Low-27-HC and High-27-HC) based on the median 27-HC concentration in bile duct tissue (see **Figure 2A**).

Total RNA extraction and RT-qPCR analysis

Total RNA was extracted from common bile duct tissue using an RNeasy Plus Mini Kit (QIAGEN K.K., Tokyo, Japan). For extraction, 50 mg (wet weight) of bile duct tissue was homogenized with a 10-fold volume of the kit lysis buffer. Five hundred μg of total RNA was used for reverse transcription (RT) to generate cDNA using a PrimeScript® RT reagent kit (TAKARA Bio Inc., Shiga, Japan). Quantitative PCR (qPCR) was performed on cDNA aliquots using FastStart DNA Master SYBR Green I and a LightCycler (Roche Diagnostics, Mannheim, Germany). PCR amplification began with a 10 min pre-incubation at 95°C, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 62°C for 10 s, and elongation at 72°C. **Table 2** shows the oligonucleotide primer sequence pairs and product sizes for each gene in the qPCR analysis. The relative concentration of PCR products from target genes was calculated using the LightCycler System software. For each run, a standard curve for each run was constructed by plotting the crossover point against the log concentration. The concentration of target molecules in each sample was calculated automatically from this curve (r = -1.00), and standardized to 18S rRNA expression. PCR product specificity was assessed through melting curve analysis.

mRNA expression levels in bile duct tissue were compared between the NC and eCCA groups and among eCCA subgroups with detectable *ERα* and *ERβ* expression (see **Figure 3A**, **3B**). Patients with eCCA were stratified into higher- and lower- expression subgroups for both genes using the Z-scores [34]. For each gene, expression values were standardized across the cohort using Z-score transformation: $Zi = (X-\mu)/\sigma$, where X is the individual value, and μ and σ represent the mean and standard deviation of the gene expression across all samples.

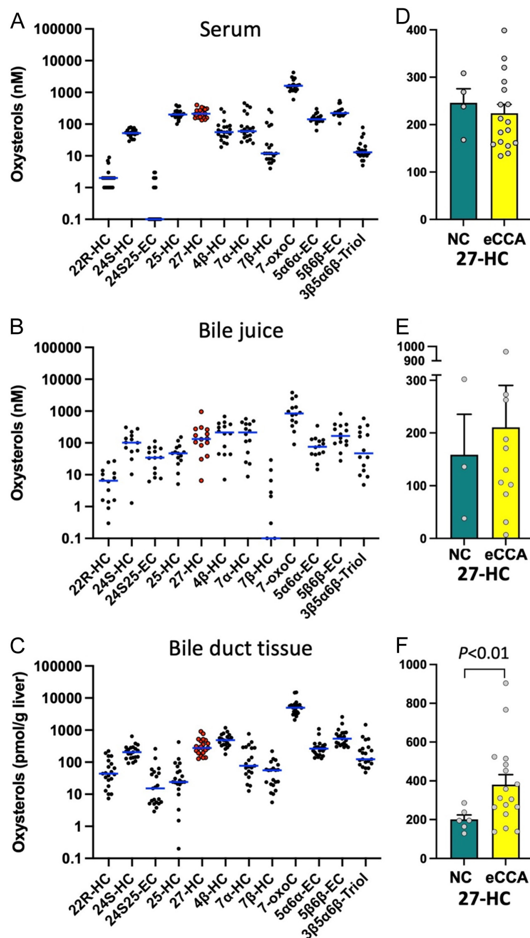


Figure 1. Oxysterol profiles and 27-HC concentrations. A-C. Oxysterol concentrations in serum (N = 21), bile (N = 14), and bile duct tissue (N = 21), respectively, from all patients. Blue bars indicate mean values; red and black dots show individual 27-HC and other oxysterol values, respectively. D-F. Comparison of 27-HC concentrations between the NC and eCCA groups in serum (N = 4 and N = 17, respectively), bile (N = 3 and N = 11, respectively), and bile duct tissue (N = 6 [four NC and two normal eCCA regions] and N = 17, respectively). Column graphs show the mean ± S.E. Statistical differences in 27-HC concentrations in bile duct tissue were analyzed using Welch's *t*-test. *Abbreviations*: *3β5α6β-Triol,* cholestane-3β,5α,6β-triol; *4β-HC*, 4β-hydroxycholesterol; *5α6α-EC*, cholesterol 5α,6α-epoxide; *5β6β-EC*, cholesterol 5β,6β-epoxide; *7-oxoC*, 7-ketocholesterol; *7α-HC,* 7α-hydroxycholesterol; *7β-HC*, 7β-hydroxycholesterol; *22R-HC*, 22R-hydroxycholesterol; *24S-HC*, 24S-hydroxycholesterol; *24S25-EC*, 24(S),25-epoxycholesterol; *25-HC*, 25-hydroxycholesterol; *27-HC*, 27-hydroxycholesterol; *eCCA*, extrahepatic cholangiocarcinoma; *NC*, non-cancer.

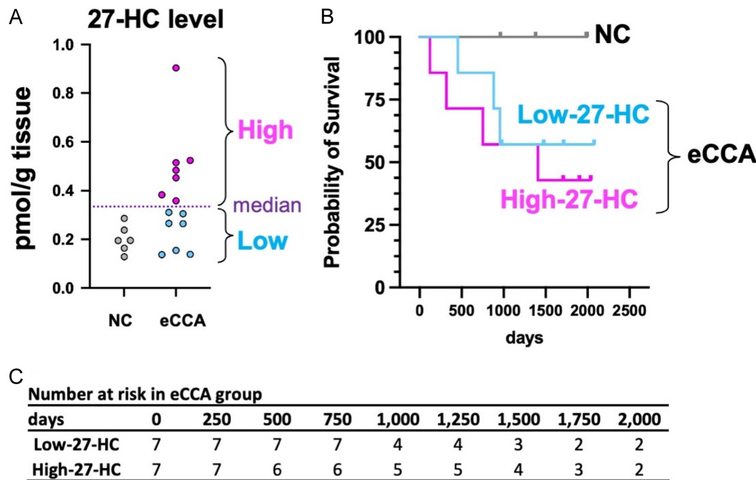
A composite expression score was calculated by summing the Zi scores of *ERα* and *ERβ*. Patients were stratified into Low-ER (N = 7) and High-ER (N = 9) subgroups according to the median composite score (see **Figure 4B**). Mortality in 14 patients with eCCA was also evaluated in the Low-ER (N = 6) and High-ER (N = 8) subgroups.

Histological analysis of surgical specimens

Common bile duct tissues were fixed in 10% neutral formalin, and embedded in paraffin. Paraffin embedded specimens were sectioned at 5 μm and used for immunohistochemical (IHC) staining with an automated system and dedicated reagents (Discovery XT system; Ventana Roche Diagnostics K.K., Basel, Switzerland). Deparaffinized specimens underwent antigen retrieval in Tris-EDTA buffer (pH 7.8; CC1, Ventana) at 95°C for 30 min and were then incubated with primary antibodies: anti-ERα (Proteintech®, Rosemont, IL, USA), anti-ERβ (Proteintech®), and anti-S100A4 (Abcam, Cambridge, UK). All antibodies were diluted 1:100 Ventana in dilution solution and applied at 37°C for 32 min. Secondary antibody incubation (37°C for 32 min) and signal detection were performed using the Ventana ultraView Universal DAB detection kit (Ventana). Counterstaining was conducted with Hematoxylin II and bluing reagents (Ventana). Specific immunoreactivity was confirmed by omitting each primary antibody. Additionally, hematoxylin and eosin (H&E) staining of paraffin-embedded specimens was performed using standard procedures.

Cell culture experiments

The ERα- and ERβ-positive human CCA-1 cell line [35, 36], derived from iCCA, was obtained from the Japanese Collection of Research Bioresources Cell Bank (National Institutes of Biomedical Innovation, Health and Nutrition, Osaka, Japan). The ERβ-positive human TFK-1 cell line [14, 36], from eCCA (TKG067), was provided by the RIKEN BioResource Research Center (Tsukuba, Japan) via the National BioResource Project of MEXT/AMED, Japan. Cell culture experiments were performed as previously described [26, 37]. CCA-1 and TFK-1 cells were seeded in 24-well plates at 2.5 × 10^4 cells per well and cultured in Ham's F-12 (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) or RPMI 1640 medium (Gibco), respectively, supplemented with 10% fetal bovine serum (FBS; Gibco) at 37°C in a humidified incubator with



Number at risk in eCCA group

days	0	250	500	750	1,000	1,250	1,500	1,750	2,000
Low-27-HC	7	7	7	7	4	4	3	2	2
High-27-HC	7	7	6	6	5	5	4	3	2

Figure 2. Survival of the eCCA patient subgroups stratified according to 27-HC concentration in the bile duct tissue. A. eCCA patients were divided into Low-27-HC (N = 7) and High-27-HC (N = 7) subgroups at the median value of 27-HC concentration. B. Survival curves for Low-27-HC, High-27-HC, and the NC (N = 4) groups. C. Number at risk every 250 days for Low-27-HC and High-27-HC subgroups. Mortality differences between eCCA subgroups were analyzed using the Log-rank Mantel-Cox test. *Abbreviations*: *eCCA*, extrahepatic cholangiocarcinoma; *NC*, non-cancer; *27-HC*, 27-hydroxycholesterol.

5% CO_2 and 95% air. After 24 h, the medium was replaced with phenol red-free RPMI 1640 containing 10% dialyzed FBS to avoid estrogenic interference from phenol red [38]. Following another 24 h of culture, cells were treated with 1, 10, 100, and 1,000 nM 27-HC (Sigma-Aldrich, St. Louis, MO, USA) or 10 nM E2 (Sigma-Aldrich) in phenol red-free RPMI-1640 medium with 10% dialyzed FBS for 48 h. Additionally, cells were treated for 48 h with the ER pan-antagonist fulvestrant (10 μM; ICI-182,780; Sigma-Aldrich) [39] or the ERβ-specific inhibitor 4-[2-phenyl-5,7-bis(trifluoromethyl) pyrazolo[1,5-a]-pyrimidin-3-yl]phenol (PHTPP; 10 μM; Sigma-Aldrich) [40], with or without 100 nM 27-HC or 10 nM E2.

To evaluate cell proliferation, images of the same central area of each well were captured with a charge-coupled device camera before and after treatments [26]. Cell numbers were counted, and the proliferation ratio was calculated by comparing post-treatment counts with baseline values. 27-HC, E2, ICI-182, 780, and PHTPP were dissolved in 0.1% ethanol, had no detectable effect on cell growth and was also added to control wells.

In CCA cell lines and the human breast cancer reference line MCF-7 (ATCC; Manassas, VA, USA), mRNA expression of ERα and ERβ was confirmed. Total RNA (500 ng) from each cell line was used for RT-qPCR, as described above. Gene expression patterns of ERα, ERβ, and 18S rRNA (housekeeping gene) were compared among these cell lines.

Statistical analyses

Data are presented as the mean ± standard error (S.E.). Differences between the two groups were assessed using unpaired Student's or Welch's t-test, depending on group variability. Multiple comparisons were evaluated using one- or two-way analysis of variance, followed by Tukey or Bonferroni post hoc tests. Mortality between eCCA subgroups was compared using the Log-rank (Mantel-Cox) test. *P* values of 0.05 were considered significant. Statistical analyses were performed using Prism 10 (GraphPad Software Inc., San Diego, CA, USA).

Results

Serum biochemical parameters and cancer stage

Serological parameters were higher in the eCCA group than in the NC group, with significant increases in bilirubin, ALP, and ALT (**Table 1**). γ-GPT, LAP, AST, CA-19-9, and CEA were also higher in the eCCA group; however, these differences were not statistically significant compared with the NC group. Cancer stages among patients with eCCA included four stage I, seven stage II, and five stage III cases (**Table 1**).

Oxysterol concentrations

Figure 1A-C illustrate oxysterol levels in serum, bile, and bile duct tissue. Substantial interindividual variability was observed, particularly in bile, likely reflecting differences in bile concentration. Levels of serum and bile 27-HC ranged from 50 to 1,000 nM (**Figure 1A**, **1B**). 7-oxoC was the most abundant oxysterol in all sample types, while 27-HC ranked second alongside

Table 2. Gene sequences of the PCR primers

Gene	Accession number		Sequence (5'-3')	Product Size (*bp*)
18S rRNA	X03205	F	GTA ACC CGT TGA ACC CCA TT	151
		R	CCA TCC AAT CGG TAG TAG CG	
CCND1	NM_053056	F	TCC TGG ATG TTG TGT GTA TCG AGA G	84
		R	ACT TGC GCG TCA CAG GAC AG	
cMYC	NM_002467	F	CCT GGT GCT CCA TGA GGA GAC	128
		R	CAG ACT CTG ACC TTT TGC CAG G	
CYP27A1	BC051851	F	CAC AAA CTC CCG GAT CAT	121
		R	AGG CTC AGA GAA GGC AGT	
EBAG9	NM_004215	F	CAC CGC AGT TGA AAT GCA TC	137
		R	GGT TTG GCC TGT ACA GTA TCT ATG A	
ERα	NM_000125	F	GAAGCTACTGTTTGCTCCTAACT	122
		R	GCAGATTCATCATGCGGAAC	
ERβ	NM_001437	F	AAG ATC GCT AGA ACA CAC CTT AC	71
		R	CGC AAC GGT TCC CAC TAA	
HIF-1α	NM_001243084	F	TAT GAG CCA GAA GAA CTT TTA GGC	145
		R	CAC CTC TTT TGG CAA GCA TCC TG	
HIF-1β	NM_001197325	F	CTG TCA TCC TGA AGA CCA GCA G	129
		R	CTG GTT CTC ATC CAG AGC CAT TC	
TFF1	NM_003225	F	GTG CAA ATA AGG GCT GCT GTT TC	178
		R	GCC GAG CTC TGG GAC TAA TCA C	
VEGFα	NM_001025366	F	TTG CCT TGC TGC TCT ACC TCC A	126
		R	GAT GGC AGT AGC TGC GCT GAT A	

Abbreviations: *18S rRNA*, 18S ribosomal ribonucleic acid; *CCND1*, cyclin D1; *cMYC*, MYC proto-oncogene (bHLH transcription factor); *CYP27A1*, cytochrome P450 27A1; *EBAG9*, estrogen receptor binding site associated antigen 9; *ERα/β*, estrogen receptor α/β; *HIF-1α/β*, hypoxia-inducible factor-1α/β; *TFF1*, trefoil factor 1; *VEGFα*, vascular endothelial growth factor α; *F*, forward; *R*, reverse; bp, base pair.

25-HC, 5α6α-EC, and 5β6β-EC in serum (**Figure 1A**); 4β-HC, 7α-HC, and 5β6β-EC in bile (**Figure 1B**); and 24S-HC, 4β-HC, 5α6α-EC, and 5β6β-EC in bile duct tissue (**Figure 1C**). 27-HC concentrations did not differ significantly between NC and eCCA groups in serum or bile (**Figure 1D**, **1E**). In contrast, 27-HC levels in bile duct tissue were significantly higher in the eCCA group than in the NC group (**Figure 1F**).

In the eCCA subgroups stratified based on bile duct 27-HC levels (**Figure 2A**), mortality was lower in the high 27-HC group than in the low 27-HC group during early and late follow-up periods, but the difference was not statistically significant (**Figure 2B**, **2C**).

Gene expression in bile duct tissue

In bile duct tissue, mRNA expression of *ERα* and *ERβ* was detected and positively correlated (**Figure 3A-C**), but did not differ significantly between the NC and eCCA groups. Similarly, other proliferation-related and ER target genes showed no significant group differences (**Figure 3D-K**). In eCCA subgroups stratified based on *ERα* and *ERβ* expression (**Figure 4A**, **4B**), mortality among 14 follow-up patients was consistently lower in the High-ER subgroup than in the Low-ER subgroup, but the difference was not statistically significant (**Figure 4C**, **4D**).

Within these subgroups, *cMYC* (**Figure 4E**), *HIF-1α* (**Figure 4F**), and *VEGFα* (**Figure 4H**) mRNA levels were significantly higher in the High-ER subgroup than in the Low-ER subgroup. *HIF-1β* expression also showed a higher tend in the High-ER subgroup, but was not statistically significant (**Figure 4G**). In contrast, the mRNA levels of *CYP27A1*, *TFF1*, *CCND1*, or *EBAG9* did not differ significantly between the two subgroups (**Figure 4I-L**).

Histological observation of ERs in bile duct tissue with eCCA

Figure 5 illustrates representative histological images of H&E staining (**Figure 5A**), S100A4 IHC (**Figure 5B**), ERα IHC (**Figure 5C**, **5D**), and ERβ IHC (**Figure 5E**, **5F**). In a bile duct tissue

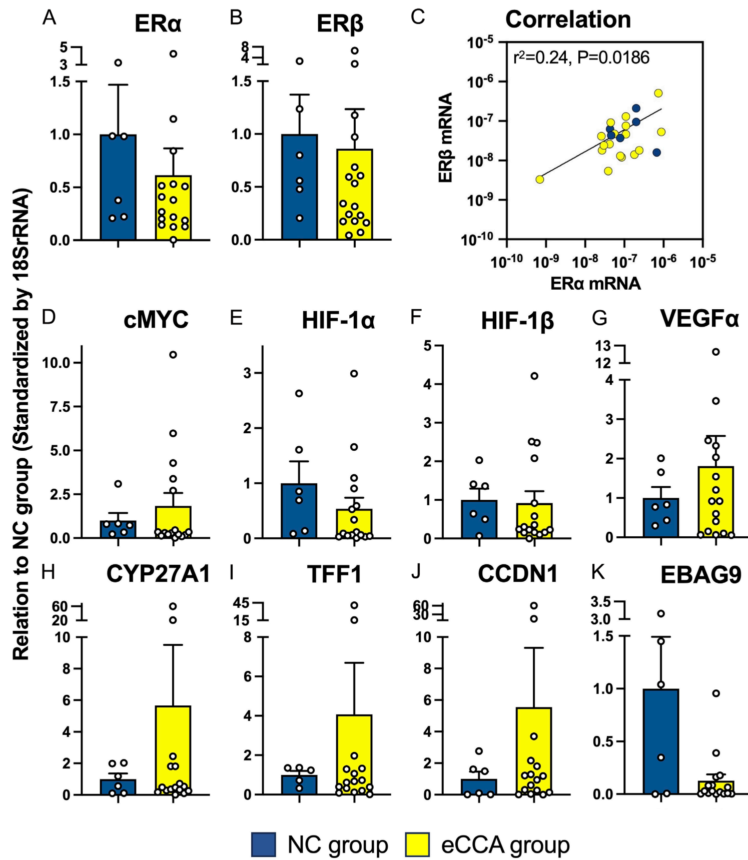


Figure 3. mRNA expression in bile duct tissue of eCCA and NC groups. A, B. mRNA expression levels of ER α and ER β. C. Correlation between ERα and ERβ mRNA levels. D-K. mRNA expression levels of cancer proliferation-related genes and ER target genes. Expression is show relative to that of the NC group after normalization to the housekeeping gene 18S rRNA. Data are presented as mean ± S.E. *Abbreviations: 18S rRNA*, 18S ribosomal ribonucleic acid; *ER*, estrogen receptor; *NC group*, non-cancer group (N = 6); *eCCA* group, extrahepatic cholangiocarcinoma group (N = 17).

specimen obtained from a female patient with eCCA exhibiting high *ERα* and *ERβ* gene expression, IHC analysis revealed protein expression of both receptors in CCA cells. In the CCA region, S100A4 protein - strongly associated with metastasis and malignancy in various tumor types - was also highly expressed.

Effect of 27-HC treatment on cell proliferation in CCA cell lines

Based on the qPCR amplification curve, mRNA expression of *ERα* and *ERβ* was detected in CCA-1 cells; however, *ERα* expression predominated over *ERβ* (**Figure 6A**), similar to that in MCF-7 cells (**Figure 6C**), a breast cancer cell line that predominantly expresses *ERα*. In contrast, the TFK-1 cell line expressed *ERβ*, but not *ERα* (**Figure 6B**).

In CCA-1 cells, proliferation increased significantly and dose-dependently after exposure to 1-1,000 nM 27-HC and by 10 nM E2 (**Figure 6D**). Proliferation increases induced by 100 nM 27-HC and 10 nM E2 were completely abolished by co-treatment with the pan-ER inhibitor ICI-182,780 (**Figure 6E**). Additionally, co-treatment with the ERβ-specific inhibitor PHTPP significantly suppressed proliferation, although its effect was weaker than that observed with ICI-182,780 (**Figure 6E**).

TFK-1 cell proliferation was also significantly and dose-dependently increased by exposure to 1-1,000 nM 27-HC and 10 nM E2 (**Figure 6F**). In this cell line, PHTPP completely inhibited the proliferative effects of 100 nM 27-HC and 10 nM E2 (**Figure 6G**). Proliferation in TFK-1 cells increased ~threefold with 27-HC and E2 compared to controls, whereas the corresponding increase in CCA-1 cells was ~1.5-fold (**Figure 6D**, **6F**).

Discussion

Studies have reported the proliferative effects of 27-HC, an endogenous ER ligand [18], in several malignancies, including breast [21-23], melanoma [24], lung [25, 26], and prostate cancers [27]. In this study, oxysterol profiles and gene expression patterns were evaluated in samples obtained from patients with eCCA. Further, the effects of 27-HC on the CCA cell line proliferation were investigated. The results demonstrated that 27-HC was detected at relatively high concentrations in serum and bile, reaching up to 1,000 nM. Moreover, 27-HC levels were significantly higher in common bile duct cancer tissues than in noncancerous bile duct tissues. Gene expression of *ERα* and *ERβ*

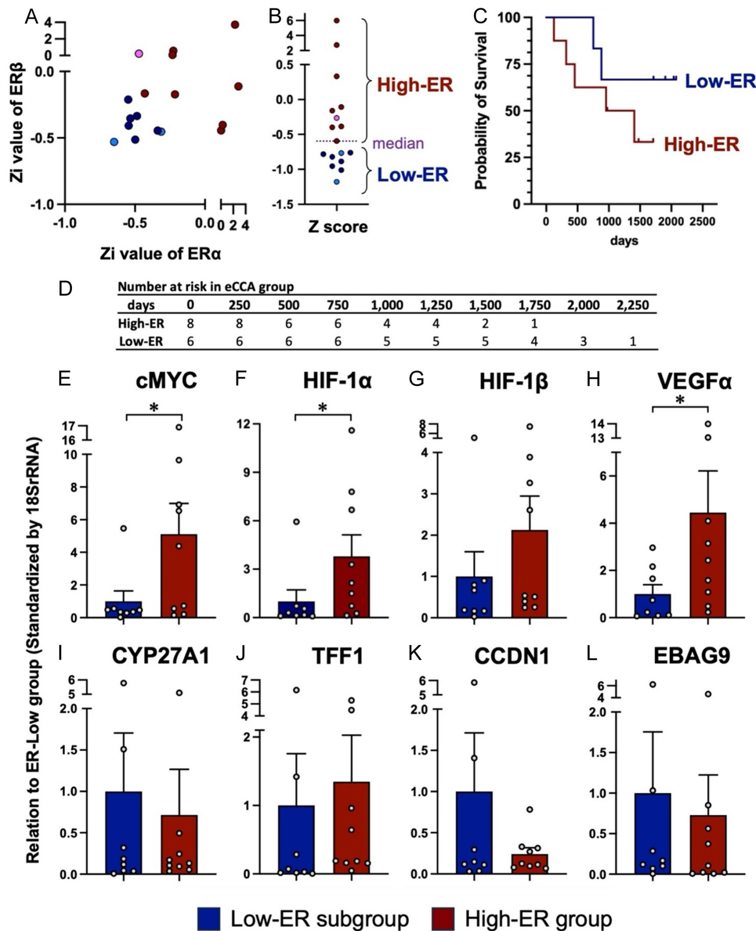


Figure 4. Survival and mRNA expression in bile duct tissues of subgroups of patient with eCCA stratified based on ER subtype mRNA levels. Subgroups were stratified based on the median of a composite expression score calculated by summing the Zi-values of *ERα* and *ERβ,* where $Zi = (X-\mu)/s$; *X*, individual value; *μ, mean;* s, standard deviation. A. Scatter plot of *ERα* and *ERβ* Zi-values in the patients with eCCA. B. *Z scores and* stratification into Low-ER and High-ER subgroups. Light pink and light blue dots indicate patients lost to follow-up in Low-ER and High-ER. C, D. Survival curves and number at risk every 250 days for Low-ER (N = 6) and High-ER (N = 8) subgroups. Mortality differences between the eCCA subgroups were analyzed using the Log-rank Mantel-Cox test. E-L. mRNA expression levels of cancer proliferation-related and ER target genes in Low-ER (N = 8) and High-ER (N = 9) subgroups of patients with eCCA, normalized to *18S rRNA* and expressed relative to Low-ER. Data are presented as the mean ± S.E. Statistical significance was determined using an unpaired Student's *t*-test or Welch's *t*-test. *Abbreviations: 18S rRNA*, 18S ribosomal ribonucleic acid; *NC*, non-cancer group; *eCCA*, extrahepatic cholangiocarcinoma; *ER*, estrogen receptor.

was confirmed in bile duct tissues, with a significant positive correlation between them. Furthermore, in CCA tissues exhibiting high *ERα* and/or *ERβ* expression, cell proliferation-related gene (*e.g., cMYC*, *HIF-1α*, and *VEGFα*) expression levels were significantly higher than those in tissues with low ER expression. These findings suggest that 27-HC, an endogenous ER ligand, promotes CCA growth via ER signaling activation. Consistent with this interpretation, 27-HC treatment at concentrations comparable to those in the serum and bile of patients with eCCA significantly and dose-dependently increased proliferation in CCA-1 cells (predominantly ERα) and TFK-1 cells (ERβ only). ER-specific inhibitors abolished 27-HC-induced proliferation confirming ER-dependent-mediated response.

Previous transcriptional activity studies demonstrated that the ligand activity of E2 toward both ERs is significantly stronger than that of 27-HC [19]. However, in this study, 27-HC induced proliferation in CCA cell lines similar to that of E2. Given that circulating 27-HC levels are 1,000-10,000 times higher than those of E2 in men and postmenopausal women, 27-HC may have a greater prominent role than E2 in ER positive CCA proliferation. Additionally, bile 27-HC may drive CCA proliferation more than circulating 27-HC. In circulation, 27-HC exists mainly as ester bound to lipoproteins, rather than in a free form [19]. In contrast, bile 27-HC exists almost entirely free in micelles composed of bile acids and phospholipids. Therefore, bile 27-HC may be taken up by CCA cells more readily than circulating 27-HC, although in bile and bile duct tissues levels showed no significant correlation (data not shown). Bile is highly concentrated in the gallbladder, and its levels vary with sampling time and site, which may explain the substantial interindividual variability in biliary oxysterols and the lack of correlation between bile and bile duct 27-HC levels. Accordingly, the primary source of 27-HC in the bile duct tissue - circulating blood, bile, or local endogenous synthesis -

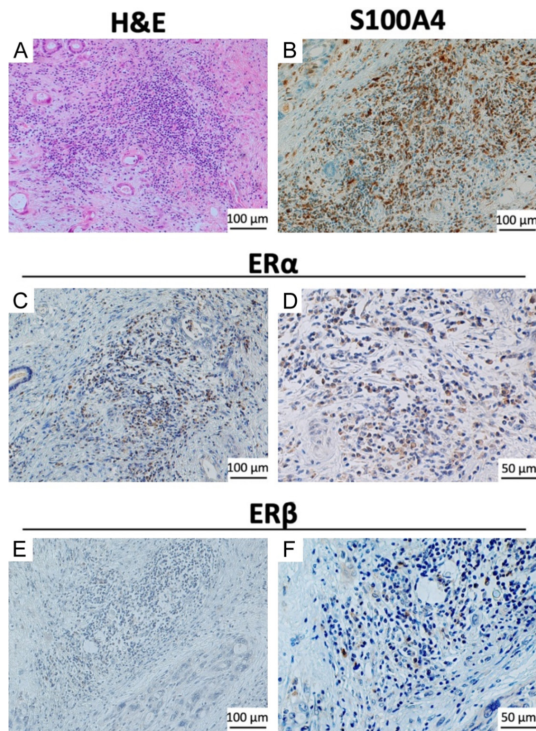


Figure 5. Histological staining of CCA tissue from a patient with eCCA. A. H&E staining (objective ×10); B. S100A4 IHC staining (×10); C, D. ERα IHC staining (×10, ×20); E, F. ERβ IHC staining (×10, ×20). The tissue specimen was obtained from a 77-year-old woman with eCCA exhibiting high *ERα* and *ERβ* gene expression. *Abbreviations: eCCA*, extrahepatic cholangiocarcinoma; *ER*, estrogen receptor; *H&E,* hematoxylin and eosin; *IHC*, immunohistochemical; *CCA*, cholangiocarcinoma.

remains unclear. Nevertheless, bile duct cells are clearly exposed to 27-HC at concentrations sufficient to activate ER signaling.

ERs, members of the steroid receptor superfamily, exist as two subtypes, ERα and ERβ, and pair as homodimers (ERα/α or ERβ/β) or heterodimers (ERα/β) [41]. In breast and prostate cancer cells, ERα and ERβ have opposing effects on cell growth: ERα promotes proliferation by activating pro-proliferative and anti-apoptotic target genes, whereas ERβ inhibits it via anti-proliferative and pro-apoptotic gene transcription [42-45]. However, studies show that ERβ, which is highly expressed in breast cancer stem cells [46], renal cell carcinoma [47, 48], bladder cancer [49], and glial tumors [50], can promote cancer progression. Consistent with these findings, we previously reported that ERβ, but not ERα, is highly expressed in lung cancer tissues compared to that in adjacent noncancerous regions in patients with non-small cell lung cancer, and that 27-HC strongly promotes proliferation in an ERβ-expressing lung cancer cell line [26]. These findings indicate that ERβ can be pro- or anti-proliferative depending on cancer type. Here, ERα and ERβ expression at the gene and protein levels were confirmed in bile duct cancer tissues from patients with eCCA. Accordingly, two human CCA cell lines were used: CCA-1, which predominantly expresses ERα, and TFK-1, which exclusively expresses ERβ. In both cell lines, 27-HC and E2 treatment significantly promoted proliferation, which was inhibited by a pan-ER inhibitor or ERβ specific inhibitor. Overall, these findings suggest that both ER subtypes promote CCA cell proliferation and that 27-HC stimulates growth via agonist activity on both ERs.

Most genes showed higher mRNA expression in the eCCA group than in the NC group, but these differences were not statistically significant, likely due to the small sample size of the NC group. Here, 27-HC and ER-related parameters in the eCCA group were compared to those in the NC group, which had small sample size insufficient for robust statistical analysis because samples from healthy individuals were unavailable. To further clarify the effect of ER expression, the eCCA group was subdivided according to ER mRNA expression levels in bile duct tissues. In this group, *cMYC*, *HIF-1α*, and *VEGFα* mRNA levels were significantly higher in the High-ER subgroup than in the Low-ER subgroup. cMYC is a master regulator of cancer progression, controlling proliferation and metabolism across diverse human cancer types, and is a direct ER target gene [51, 52]. HIF-1α, a transcription factor regulating angiogenic factors, is generally induced by hypoxia, but ER activation via the phosphatidylinositol 3-kinase pathway also promotes its translation [53]. *VEGFα* gene expression is regulated by cooperative ER and HIF-1α binding to its promoter [53]. Similarly, *cMYC, HIF-1α*, and *VEGFα* gene expression in CCA tissue appears to be directly or indirectly regulated by ER activation. These subgroups did not differ significantly in the expression patterns of other ER target genes (*TFF-1*, *CCND1*, and *EBAG9*). Since ER target gene expression depends on ER levels and availability of ligands, including E2 and 27-HC, the lack of differences in these genes may

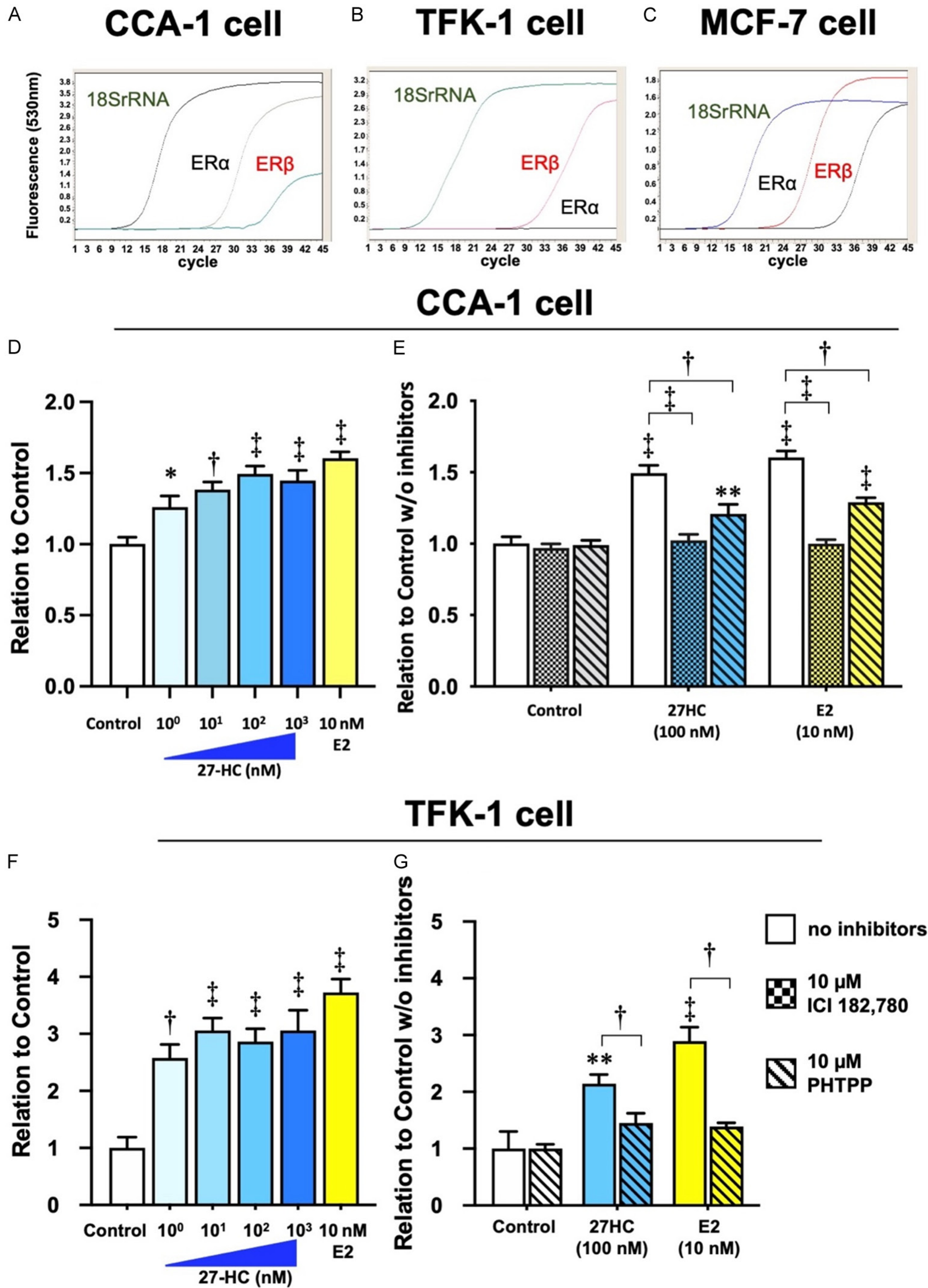


Figure 6. Effects of 27-HC on cell proliferation and ER signaling in CCA-1 and TFK-1 cells. A-C. RT-qPCR amplification curves for ERα, ERβ, and 18S rRNA in human *CCA* cell lines; CCA-1 and TFK-1, and human breast cancer cell line; MCF-7, as reference. Threshold cycles: CCA-1, ERα 25.8, ERβ 32.3, and 18S rRNA 15.5; MCF-7, ERα 20.8, ERβ 31.7, and 18S rRNA 10.5; TFK-1, ERβ 28.8 and 18S rRNA 10.8. D, F. CCA-1 and TFK-1 cells seeded at a density of

2.5×10^4 cells/well in 24-well plates and treated with various 27-HC concentrations or 10 nM E2, with or without ER inhibitors, in phenol red-free medium for 48 h (N = 5 for CCA-1 and N = 7 for TFK-1). E, G. Effects of pan-ER inhibitor ICI-182,780 (10 μM) and ERβ-specific inhibitor PHTPP (10 μM) cells treated with 100 nM 27-HC or 10 nM E2 for 48 h (N = 5 for CCA-1, N = 8 for TFK-1). Cell proliferation is expressed relative to respective controls (0.1% ethanol). Data are presented as the mean ± S.E., Statistical significance was performed using one- or two-way analysis of variance with Bonferroni's post hoc multiple-comparison. *$P<0.05$, **$P<0.01$, †$P<0.001$, and ‡$P<0.0001$ denote comparison among identical conditions or with controls. *Abbreviations: 18S rRNA*, 18S ribosomal ribonucleic acid; *27-HC*, 27-hydroxycholesterol; *CCA*, cholangiocarcinoma; *E2*, 17β-estradiol; *ER*, estrogen receptor; PHTPP, 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]-pyrimidin-3-yl]phenol; *RT-qPCR*, reverse transcription quantitative PCR.

reflect complex interactions among multiple regulatory factors.

In the eCCA subgroups, the High-ER subgroup had a lower mortality rate than the Low-ER subgroup over a maximum postoperative follow-up period of 2,708 days, but the difference was not statistically significant. This is likely due to the small number of patients within the cohort. The low survival trend in the High-ER eCCA subgroup supports our hypothesis and confirms that CCA tissue strongly associates with CAA progression. Therefore, further studies in larger cohorts are warranted.

Hypercholesterolemia increases the risk of ERα-positive breast cancer [21]. To date, no clear evidence indicates that hypercholesterolemia increases the risk of CCA. However, a study reports that dyslipoproteinemia increases the risk of iCCA and eCCA [54]. Furthermore, a recent systematic review and meta-analysis report that statin use is associated with a lower CCA risk in patients with iCCA and eCCA [55]. Statins decrease blood and bile cholesterol [56] and likely reduce 27-HC levels [57]. Therefore, statin-induced in 27-HC reductions may partly explain the decrease in CCA risk.

Here, we were unable to obtain an eCCA cell line expressing only ERα wihtout ERβ. Therefore, direct assessment of the effect of 27-HC on eCCA cell proliferation through ERα alone was not possible. However, studies report that 27-HC promotes proliferation of various cancer cells via ERα [22], suggesting a similar mechanism in ERα-positive eCCA. Accordingly, if eCCA tissues collected through biopsy or surgery show high expression of either ER subtype, anti-estrogen therapy may serve as a potential adjuvant treatment.

In conclusion, bile duct tissue from patients with eCCA contained significantly higher 27-HC levels, which exhibit SERM-like activity compared to noncancerous tissues. This study shows that CCA tissues express ERα and ERβ and that 27-HC promotes proliferation in human CCA cell lines expressing these receptors. Collectively, these findings suggest that 27-HC within the tumor microenvironment promotes proliferation of ER-expressing eCCA cells. Thus, targeting 27-HC and ER signaling may offer promising therapeutic strategies for CCA.

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Disclosure of conflict of interest

None.

Abbreviations

18S rRNA, 18S ribosomal ribonucleic acid; *22R-HC*, 22R-hydroxycholesterol; *24S-HC*, 24S-hydroxycholesterol; *24S25-EC*, 24(S),25-epoxycholesterol; *25-HC*, 25-hydroxycholesterol; *27HC*, 27-hydroxycholesterol; *3β5α6β-Triol*, cholestane-3β,5α,6β-triol; *4β-HC*, 4β-hydroxycholesterol; *5α6α-EC*, cholesterol 5α,6α-epoxide; *5β6β-EC*, cholesterol 5β,6β-epoxide; *7-oxoC*, 7-ketocholesterol; *7α-HC*, 7α-hydroxycholesterol; *7β-HC*, 7β-hydroxycholesterol; *ALP,* alkaline phosphatase; *ALP,* alkaline phosphatase; *ALT*, alanine aminotransferase; *AST*, as-

partate aminotransferase; *C*, cisplatin; *CA-19-9*, carbohydrate antigen19-9; *CCND1*, cyclin D1; *CCA*, cholangiocarcinoma; *CEA*, carcinoembryonic antigen; *cMYC*, MYC proto-oncogene, bHLH transcription factor; *CYP27A1*, cytochrome P450 27A1; *E2*, 17β-estradiol; *EBAG9*, estrogen receptor binding site associated antigen 9; *eCCA*, extrahepatic cholangiocarcinoma; *ER*, estrogen receptor; *FBS*, fetal bovine serum; *G*, gemcitabine; *γ-GPT*, γ-glutamyl transpeptidase; *H&E*, hematoxylin and eosin; *HIF-1*, hypoxia-inducible factor-1; *iCCA*, intrahepatic cholangiocarcinoma; *IHC stain*, immunohistological stain; *LAP*, leucine aminopeptidase; *NC*, non-cancer; *PHTPP*, 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]-pyrimidin-3-yl]phenol; *qPCR,* quantitative PCR; *RT*, reverse transcription; *S.E.*, standard error; *SERM*, selective estrogen receptor modulator; *TFF1*, trefoil factor 1; *VEGFα*, vascular endothelial growth factor α.

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