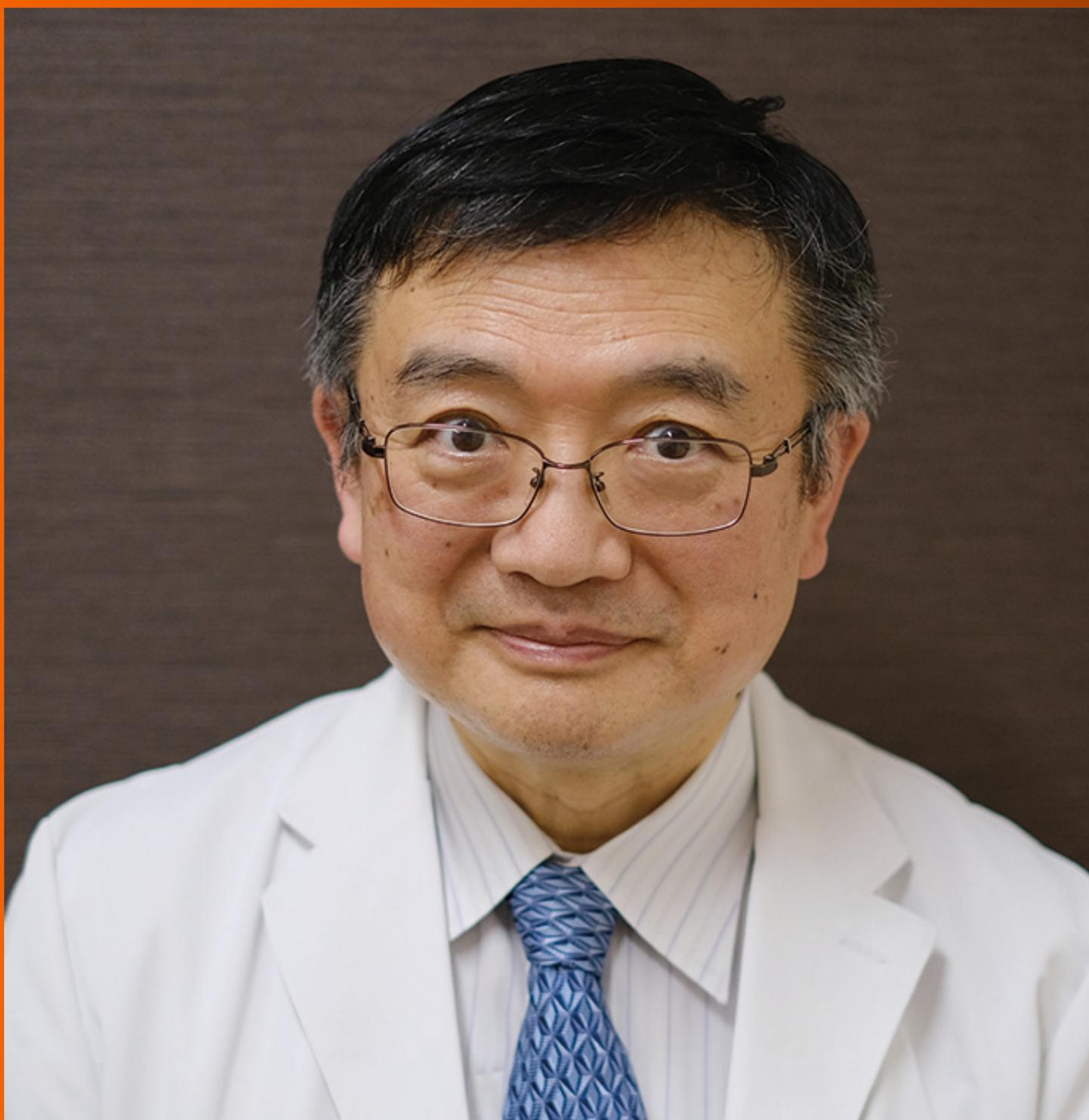


World Journal of *Gastroenterology*

World J Gastroenterol 2023 July 7; 29(25): 3932-4098



REVIEW

- 3932 Glycogen storage diseases: An update
Gümüř E, Özen H
- 3964 Past, present, and future of long-term treatment for hepatitis B virus
Broquetas T, Carrión JA
- 3984 Impact of gut microbiome in the development and treatment of pancreatic cancer: Newer insights
Bangolo AI, Trivedi C, Jani I, Pender S, Khalid H, Alqinai B, Intisar A, Randhawa K, Moore J, De Deugd N, Faisal S, Suresh SB, Gopani P, Nagesh VK, Proverbs-Singh T, Weissman S

MINIREVIEWS

- 3999 Insulin resistance and adipose tissue interactions as the cornerstone of metabolic (dysfunction)-associated fatty liver disease pathogenesis
Pal SC, Méndez-Sánchez N
- 4009 Recent advances and current challenges in endoscopic resection with the full-thickness resection device
Mun EJ, Wagh MS
- 4021 Dynamic contrast enhanced ultrasound in gastrointestinal diseases: A current trend or an indispensable tool?
Paratore M, Garcovich M, Ainora ME, Riccardi L, Gasbarrini A, Zocco MA
- 4036 Fractal nature of human gastrointestinal system: Exploring a new era
Grizzi F, Spadaccini M, Chiriva-Internati M, Hegazi MAAA, Bresalier RS, Hassan C, Repici A, Carrara S

ORIGINAL ARTICLE**Basic Study**

- 4053 Molecular profiling reveals potential targets in cholangiocarcinoma
Liu D, Shi Y, Chen H, Nisar MA, Jabara N, Langwinski N, Mattson S, Nagaoka K, Bai X, Lu S, Huang CK

Retrospective Study

- 4072 Non-invasive model for predicting high-risk esophageal varices based on liver and spleen stiffness
Yang LB, Gao X, Li H, Tantai XX, Chen FR, Dong L, Dang XS, Wei ZC, Liu CY, Wang Y

Observational Study

- 4085 Real-world effectiveness and safety of direct-acting antivirals in hepatitis C virus patients with mental disorders
Dybowska D, Zarębska-Michaluk D, Rzymiski P, Berak H, Lorenc B, Sitko M, Dybowski M, Mazur W, Tudrujek-Zdunek M, Janocha-Litwin J, Janczewska E, Klapaczyński J, Parfieniuk-Kowerda A, Piekarska A, Sobala-Szczygieł B, Dobrowolska K, Pawłowska M, Flisiak R

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Basic Study

Molecular profiling reveals potential targets in cholangiocarcinoma

Dan Liu, Yang Shi, Hongze Chen, Muhammad Azhar Nisar, Nicholas Jabara, Noah Langwinski, Sophia Mattson, Katsuya Nagaoka, Xuewei Bai, Shaolei Lu, Chiung-Kuei Huang

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Abstract**BACKGROUND**

Cholangiocarcinoma (CCA) is a devastating malignancy and has a very poor prognosis if tumors spread outside the liver. Understanding the molecular mechanisms underlying the CCA progression will likely yield therapeutic approaches toward treating this deadly disease.

AIM

To determine the molecular pathogenesis in CCA progression.

METHODS

In silico analysis, *in vitro* cell culture, CCA transgenic animals, histological, and molecular assays were adopted to determine the molecular pathogenesis.

RESULTS

The transcriptomic data of human CCA samples were retrieved from The Cancer Genome Atlas (TCGA, CHOL), European Bioinformatics Institute (EBI, GAD-00001001076), and Gene Expression Omnibus (GEO, GSE107943) databases. Using Gene set enrichment analysis, the cell cycle and Notch related pathways were demonstrated to be significantly activated in CCA in TCGA and GEO datasets. We, through differentially expressed genes, found several cell cycle and notch associated genes were significantly up-regulated in cancer tissues when compared

with the non-cancerous control samples. The associated genes, *via* quantitative real-time PCR and western blotting assays, were further examined in normal human cholangiocytes, CCA cell lines, mouse normal bile ducts, and mouse CCA tumors established by specifically depleting P53 and expressing Kras^{G12D} mutation in the liver. Consistently, we validated that the cell cycle and Notch pathways are up-regulated in CCA cell lines and mouse CCA tumors. Interestingly, targeting cell cycle and notch pathways using small molecules also exhibited significant beneficial effects in controlling tumor malignancy. More importantly, we demonstrated that several cell cycle and Notch associated genes are significantly associated with poor overall survival and disease-free survival using the Log-Rank test.

CONCLUSION

In summary, our study comprehensively analyzed the gene expression pattern of CCA samples using publicly available datasets and identified the cell cycle and Notch pathways are potential therapeutic targets in this deadly disease.

Key Words: Bile duct cancer; Notch; Cell cycle; Liver cancer; Therapeutic target

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Core Tip: Molecular profiling of cholangiocarcinoma (CCA) has been conducted using various cohorts. However, the identified targets vary among different cohorts. In the current study, we combined different cohorts of CCA RNA sequencing datasets and refined the potential therapeutic targets in human CCA malignancy. We validated the findings using human CCA cell lines, the Kras^{G12D} and P53 mutation transgenic mouse model, and human CCA clinical data, thus supporting the potential of targeting the identified pathways in clinical trials.

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INTRODUCTION

Cholangiocarcinoma (CCA) is a devastating malignancy with “silent” clinical manifestations, genetically heterogeneous, anatomically distinct, but limited treatment options[1]. CCA can be classified as three subtypes depending on the origin of anatomical site, including intrahepatic (iCCA), perihilar (pCCA) and distal (dCCA) CCA[2]. If CCAs are diagnosed at the early stage, surgical resection may be a treatment option. However, CCA tumors are generally diagnosed at advanced stages due to lack of symptoms. Therefore, it is urgent to develop early detection markers and effective therapies for CCA patients. Understanding the molecular pathogenesis of CCAs may help identify early detection markers and potential therapies for this deadly disease.

Advances in whole-exome and transcriptome sequencing have paved the way for precision medicine in patients with advanced-stage or unresectable lesions[3-5]. Several genetic mutations have been identified in CCA patients[6]. Among them, IDH1 mutations have been well studied and the IDH1 mutation specific inhibitor has been developed for targeting malignant tumors with this mutation. In the ClarIDHy clinical trial, it was found that Ivosidenib-IDH1 mutation inhibitor could improve CCA patient overall survival (OS) and progression free survival[7,8], which eventually led to the FDA approval of Ivosidenib use in CCA patients with IDH1 mutation. Nevertheless, the 5-year survival of these patients treated with Ivosidenib is not significantly improved[7,8]. Besides, there is no effective therapy for those CCA patients with wild-type IDH1, despite that cisplatin and gemcitabine could extend the median OS for 3.6 mo but is not curative. Therefore, there is still an unmet need for identifying effective therapies in CCA patients.

In the current study, we utilized the transcriptomic profiling of CCA to explore the oncogenic driver events, analyzed the relevant genes expression, and identified the feasibility of target therapies with respect to those tumorigenic lesions.

MATERIALS AND METHODS

Human data

A total of 2 archival tissue blocks from patients with eCCA were retrieved from the First Affiliated Hospital of Zhengzhou University (Zhengzhou, Henan Province, China) and the specimens were independently diagnosed by two hepatobiliary pathologists. This study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of the same institution (Approval No. 2022-KY-1514-002). Informed consents were obtained from all patients.

Cell Lines and reagents

Human CCA cell lines, including H1 (OCUCh-LM1-H1)[9], HuCCT1[10], SSP-25[11], and ETK1[12] cell lines were kindly provided by Dr. Jack Wands. TFK-1 and RBE were purchased from RIKEN Cell Bank (Tsukuba, Japan). The non-malignant human bile duct cells hBD was purchased from Celprogen Inc (Torrance, CA, United States, Catalog Number: 36755-12). CCA cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS; Gibco™, Thermo Scientific, Waltham, MA, United States; Cat# 10099141C), 100 µg/mL streptomycin, and 100 U/mL penicillin (HyClone™, Logan, UT, United States). The hBD cell line was grown in DMEM containing 10% FBS, 100 µg/mL strep. All cell lines were authenticated by STR and were negative for contamination of mycoplasma recently. Arcyriaflavin A, Flavopiridol, Roscovitine, and γ -secretase inhibitor, DAPT (N-[N-(3, 5-difluorophenacetyl)-l-alanyl]-s-phenylglycine-butyl ester) were all purchased from Cayman Chemical (Ann Arbor, MI, United States).

Data collection

RNA-Seq, DNA methylation, and clinicopathological data of 36 patients with cholangiocarcinoma (liver/normal bile duct = 9, iCCA = 30, pCCA = 4, dCCA = 2) were retrieved from The Cancer Genome Atlas (TCGA) database. The Genomic Data Commons mRNA quantifications were calculated by STAR [13], and then annotated by the reference gene model GENCODE v36. iCCA and the related pancreatic normal livers from 30 patients, and their survival information were employed (GSE107943) [14]. Fastq files of RNA-Seq from 162 samples of CCA (iCCA = 122, pCCA = 14, dCCA = 26) and the associated survival status of patients were fetched from the European Genome-phenome Archive[15].

Survival analysis

The Kaplan-Meier analysis was performed to evaluate the OS and disease-free survival (DFS) of patients from GEO and EBI cohorts. The optimal cutoff point for two groups was estimated based on maximally selected rank statistics[16], and then significance of survival analysis was calculated using log-rank test between the two groups.

Gene set enrichment analyses

Gene set enrichment analysis (GSEA) was conducted using R package “Pi”[17]. Hallmark (H) and KEGG subset of Canonical Pathways (CP) gene sets were selected for GSEA[18].

Tumor-infiltrating immune cell analysis

Single-sample GSEA was performed to analyze the tumor-infiltrating lymphocytes (TILs) based on the 28 immune cell types[19]. Correlations between the indicated genes expression and each infiltrating immune cells levels were calculated based on Spearman correlation (ρ).

Genetically engineered mouse

All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Rhode Island Hospital, and all experiments were conducted in accordance with the guidelines of this IACUC. The specific Kras^{G12D} (LSL-Kras^{G12D}), floxed-p53 (p53L/L), and albumin-Cre mutants strains were purchased from the Jackson Laboratory (Bar Harbor, ME, United States). They were intercrossed to achieve the mutant mice as indicated in the results section. The specific primers and genotyping protocol were performed following the instruction suggested by the Jackson Laboratory. Mice were bred in a pathogen-free environment and provided with free access to food and sterilized water *Ad Libitum*. Animals within experimental cohorts were monitored until the appearance of illness, including diminished activity, abdominal bloating, and cachexia, followed by full necropsy and the subsequent histopathological analysis.

MTT cell growth assay

Three CCA cell lines, including HuCCT1, H1, RBE, were seeded at 2×10^3 cells/well into 96-well plate in six replicates. And then the above-mentioned cells were treated with small molecular inhibitors targeting cell cycle (Arcyriaflavin a, Flavopiridol, and Roscovitine) and Notch (DAPT) pathways 24 h post-seeding, respectively. The treatments were changed as indicated at days 1, 3, 5, and 7. Cell proliferation was measured using MTT at the following day (day 0 and day 1) and every 48 h after (up to day

7). Absorbance at OD570 nm and 650 nm were read and then normalized to vehicle control (0.1% DMSO or the corresponding medium). The relative cell proliferation rates were calculated as OD570-OD650.

RNA extraction and real-time quantitative PCR

Total RNA was extracted from CCA cell lines using TRIzol™ (Invitrogen, Thermo Scientific; Cat# 15596026) according to the manufacturer's protocol. 1 µg of total RNA was used to reverse transcription using iScript™ Reverse Transcription Supermix (Bio-Rad, Hercules, CA, United States, Cat#1708840). The qRT-PCR was conducted using SYBR® Green Realtime PCR Master Mix (Bio-Rad, Cat#1725270) by QuantStudio™ 5 Real-Time PCR System (Applied Biosystems). The relative mRNA expression was calculated based on 2^{-ΔΔCt} protocol. All primers were listed in [Supplementary Table 1](#).

Western blotting

Whole-cell protein extractions was acquired using RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, Cat# 89901), supplemented with protease inhibitor (Thermo Fisher Scientific, Cat#78430). The concentration of protein was quantified using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Cat# J63283.QA) before mixed with protein loading buffer (3×). Then the protein samples were separated by 10% SDS-PAGE, and the detailed procedures conducted as previously described[20].

Antibodies including α-Tubulin, GAPDH, Cyclin D1, P21, PCNA, E2F1, CDK2, CDK4, CDK6, RB1, JAG1, JAG2, Caspase 8, BAX, XIAP, BCL-2L12, ATR, ATM, Rad50, Mre11, PIK3C3, LC3A/B, and ULK1 were purchased from Cell Signaling Technology (Danvers, MA, United States) and Santa Cruz Biotechnology (Dallas, TX, United States).

Statistical analysis

Statistical analyses were performed using STATA software 16.0 (StataCorp, College Station, TX, United States) and R software (version 4.2.0). Statistical analysis was performed using unpaired Student's *t* and Mann-Whitney *U* test with respect to Gaussian distribution or not, respectively, unless otherwise indicated. Kruskal-wallis test was performed to compare the expression of genes examined by PCR, and the *post-hoc* tests within two groups were adjusted by Holm's correction. Spearman correlation coefficient (ρ) is used to evaluate the correlations between DNA methylation levels of CpG islands and the associated genes expression. Wilcoxon signed-rank test was utilized to compare the expression level of cell cycle and Notch related genes between iCCA and its corresponding normal tissues. Two-way repeated measures Anova was utilized to compare optical densities of CCA cell lines treated with the serial gradient concentration of drugs, which measured more than once. Then simple effect at day 7 was calculated when there were interaction effects between time and dosages. *P* values were shown as: ^a*P* < 0.05, ^b*P* < 0.01, and ^c*P* < 0.001.

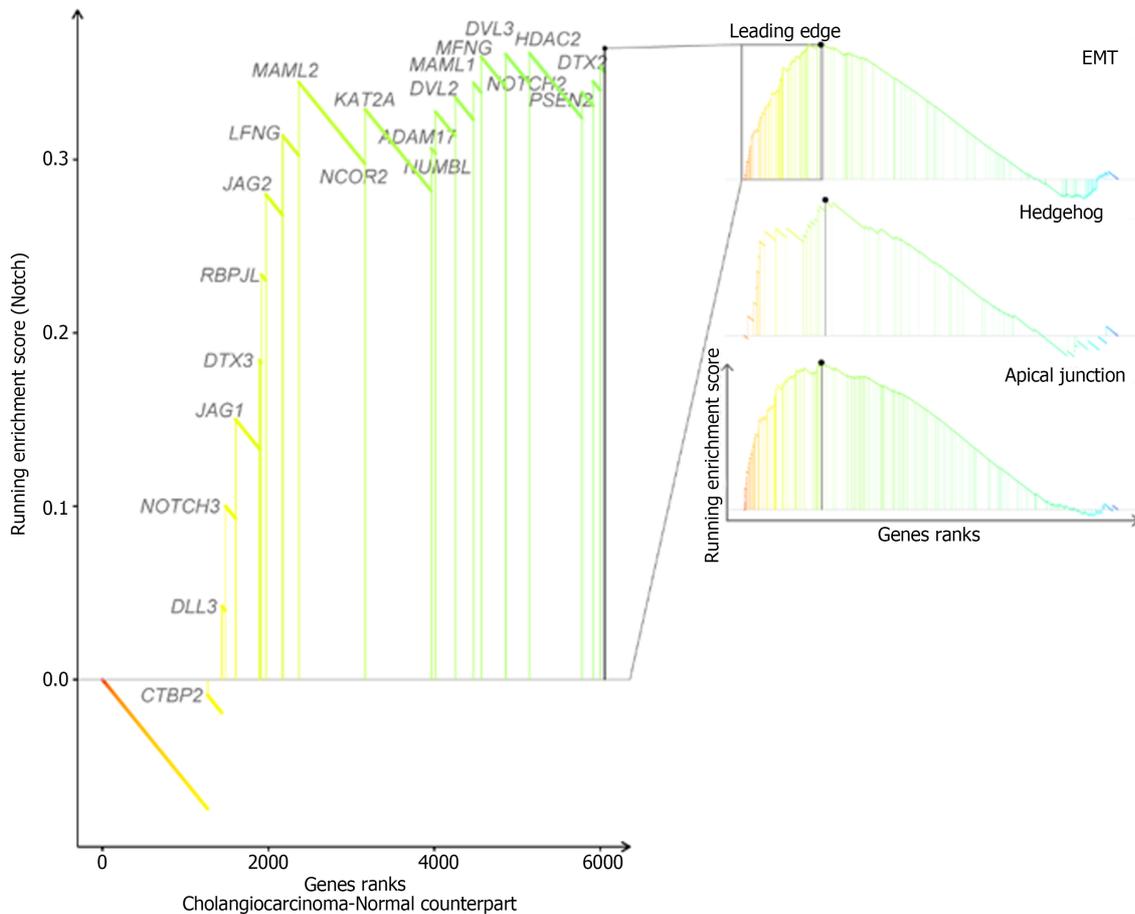
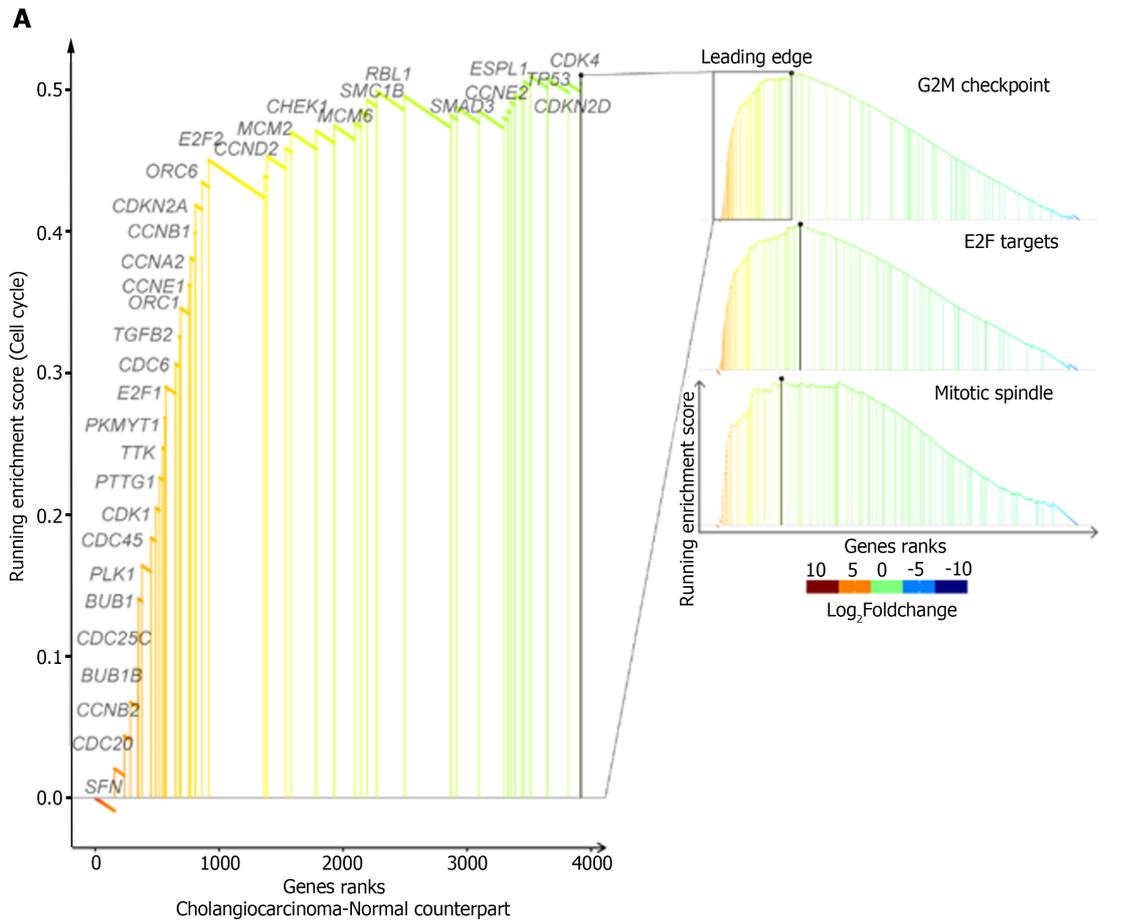
RESULTS

CCA oncogenesis is highly associated with elevated cell cycle and Notch associated pathways

To determine what molecular events are up-regulated in cholangiocarcinogenesis, we downloaded the transcriptomic data of CHOL from the TCGA database. We performed the GSEA and found that cell cycle associated pathways were preferentially activated in tumors compared with the normal samples, such as genes involved in G2M checkpoint, E2F Targets, and Mitotic spindle ([Figure 1A](#), upper). Besides, the pathways related with Notch were also induced in tumors, *e.g.*, genes implicated in epithelial mesenchymal transition (EMT), Hedgehog, and Apical Junction ([Figure 1A](#), lower). We retrieved the leading edge gene sets from those pathways, which drive the enrichment score and dominate the tumorigenicity of those pathways. Consistently, the genes from those leading edges are distinctly up-regulated in tumors compared with the normal samples, in line with the oncogenic properties ([Figure 1B](#) and [C](#)). Together, through the analysis of GSEA and differentially expressed genes, we found cell cycle and Notch pathways are up-regulated in CCA patients compared to non-malignant patient controls.

Cell cycle and Notch associated pathways were found upregulated in CCA cell lines compared to normal biliary epithelial cells

To facilitate the application of identified genes in preclinical studies, we must validate if these findings are reproducible in established normal biliary epithelial cells and CCA cells. In this case, we would be able to evaluate whether these pathways can be targeted using the *in vitro* cell culture system. Thus, we examined several leading-edge genes associated with these pathways in normal biliary epithelial cells and several CCA cell lines. The results suggest that several cell cycles associated genes were up-regulated in CCA cancer lines, including PCNA, E2F1, CDK2, CDK4, and CDK6 ([Figure 2A](#)). Besides, JAG1 and JAG2 which serve as the Notch ligand also substantially increased in CCA cell lines when compared with normal biliary epithelial cells ([Figure 2B](#)). We also examined several genes involved in autophagy, cell death, and DNA damage pathways since we wanted to illustrate if cell cycle associated



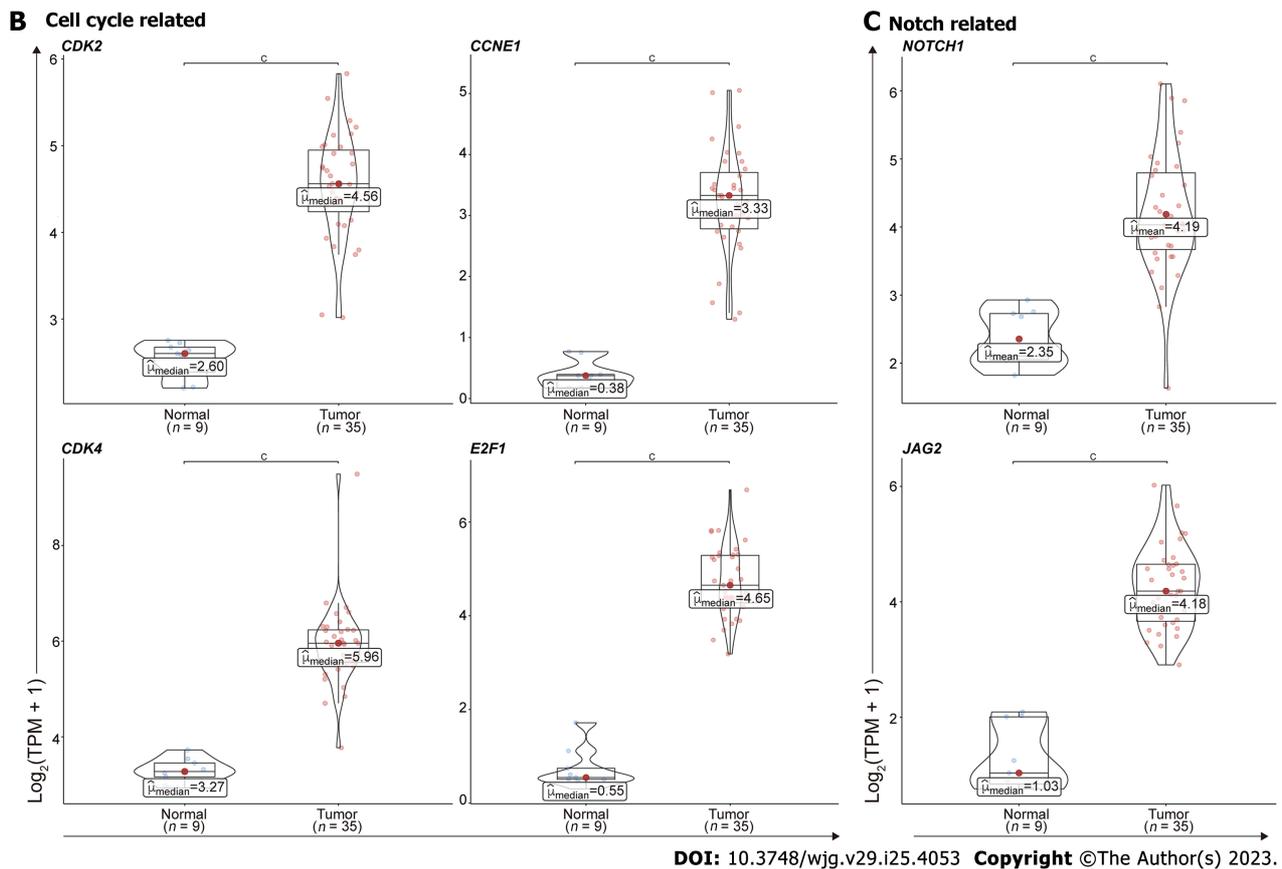


Figure 1 Cholangiocarcinogenesis driven by cell cycle and Notch associated pathways. A: Rank-based gene set enrichment analysis of genes preferentially activated in tumors identified the tumorigenic pathways, cell cycle and Notch from Hallmark gene sets, in CHOL transcriptome from TCGA database. Upper: Cell cycle associated pathways, including G2M checkpoint, E2F Targets, and Mitotic spindle gene sets; Lower: Notch associated pathways, including epithelial mesenchymal transition, Hedgehog, and apical junction gene sets. The mRNA expression levels from the leading edges of cell cycle (B) and Notch (C) associated pathways were analyzed in human normal bile ducts ($n = 9$) and CCA samples ($n = 35$) using TCGA database, suggesting up-regulation of cell cycle and Notch associated genes in CCA samples. TPM: Transcripts per kilobase million; Red: Cholangiocarcinoma (iCCA/eCCA), Blue: Normal bile ducts and/or paracancerous liver; EMT: Rptithelial mesenchymal transition. $^{\circ}P < 0.001$ when compared with the normal control.

pathways play a dominant role or cell death pathways dominate the CCA tumorigenesis. Interestingly, we found several up-regulated genes that are associated with DNA damage and cell death pathways, but not autophagy (Figure 2C-E). Moreover, the negative correlations between the methylation status (β value) and its expression of those genes suggested that the aberrant expression might be attributed to the hyper- or hypomethylation (Supplementary Figure 1).

Up-regulated cell cycle and Notch associated gene expression levels were correlated with protein expression profiles in CCA cell lines

To determine if the identified genes have increased protein expression levels or promote other protein expression levels of the same pathway in CCA cell lines compared with the control biliary epithelial cells, we decided to examine protein expression of several major representative genes in each pathway (Figure 3, Supplementary Figure 2). As shown, several cell cycles associated genes were up-regulated in CCA cell lines when compared with the control biliary epithelial cells, including CDK2, CDK4, CDK6, and E2F1. Unexpectedly, several cyclins which include cyclin D1 and cyclin E1 were found decreased (Figure 3A). When we examined Notch ligands which include JAG1 and JAG2, we found JAG1 and JAG2 were all significantly increased in CCA cell lines compared to the biliary epithelial cells (Figure 3B). We further examined autophagy associated proteins and found no significant difference in autophagy between normal and malignant cells based on the autophagy definition (Figure 3C)[21]. In line with the mRNA change data, several apoptosis and DNA damage associated genes that were identified elevated in CCA cell lines were found increased as well (Figure 3D and E). Together, the mRNA and protein profile data demonstrated that it is feasible to identify potential target genes through mining public datasets and validating through *in vitro* human CCA cell lines.

Validation of the identified genes in a triple transgenic CCA mouse model

It has been well characterized that *KRAS* and *P53* are two of the most mutant genes in human CCA tumor samples[22]. Thus, a previous study has established the CCA transgenic mouse model by

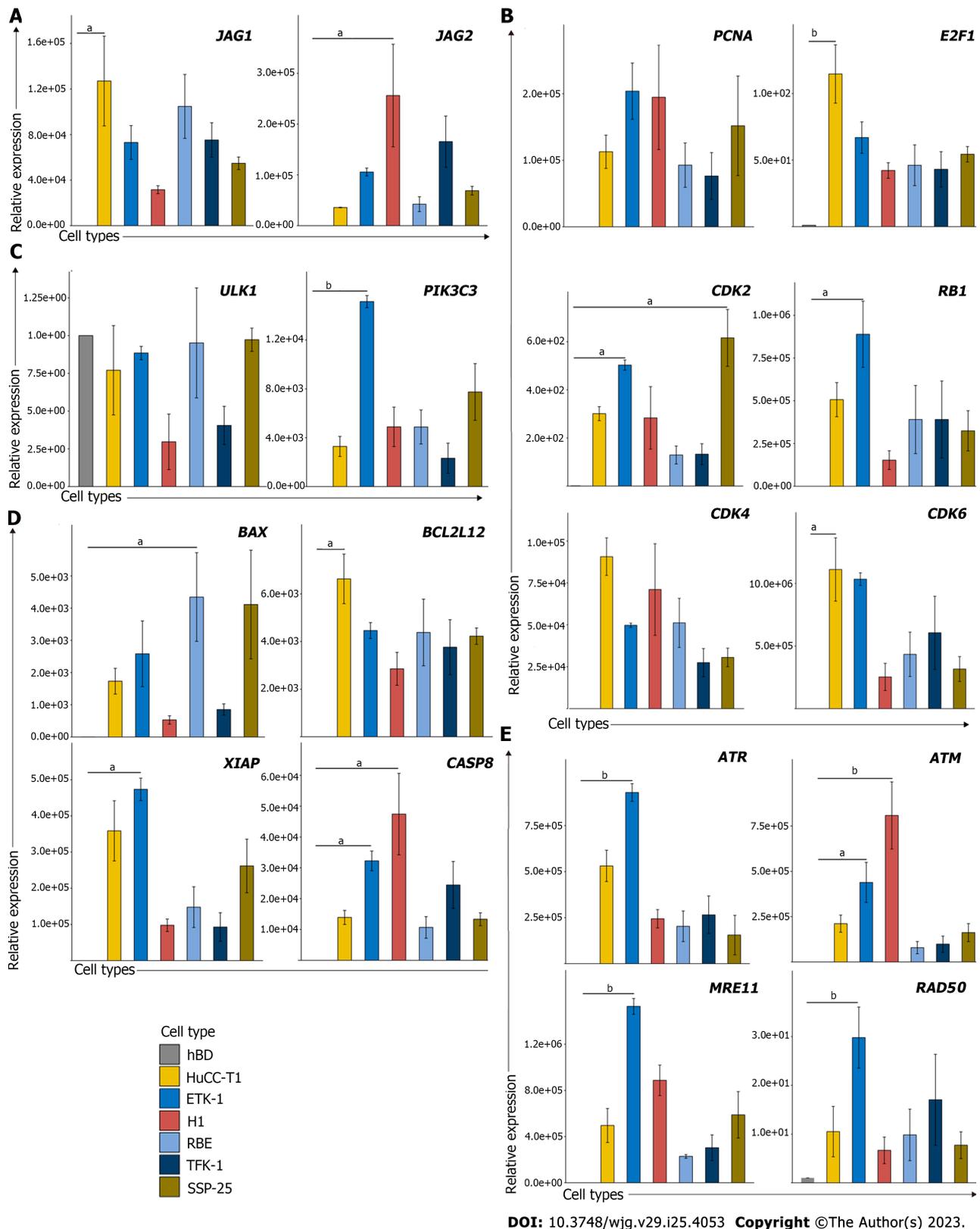


Figure 2 Cell cycle and Notch associated pathways are increased in cholangiocarcinoma cell lines *in vitro*. The mRNA expression levels of leading-edge genes from (A) Notch, (B) cell cycle, (C) autophagy, (D) cell death, and (E) DNA damage were examined in cholangiocarcinoma (CCA) cell lines compared to normal biliary epithelial cells (hBD), indicating up-regulation of these genes in CCA tumor samples. CCA tumor cell lines including HuCC-T1, ETK-1, H1, RBE, TFK-1 and SSP25. Data are relative to hBD cell line and normalized to 18S rRNA.

conditionally activating $Kras^{G12D}$ and depleting P53 (either P53 homozygous or P53 heterozygous knockout) in mouse liver using the Albumin promoter driven cre recombinase[23]. We also established the CCA transgenic mouse (Figure 4A) as evidenced by the genotyping data showing positive cre, $Kras^{G12D}$, and heterozygous floxed P53 genes (Figure 4B). The gross images of representative mouse

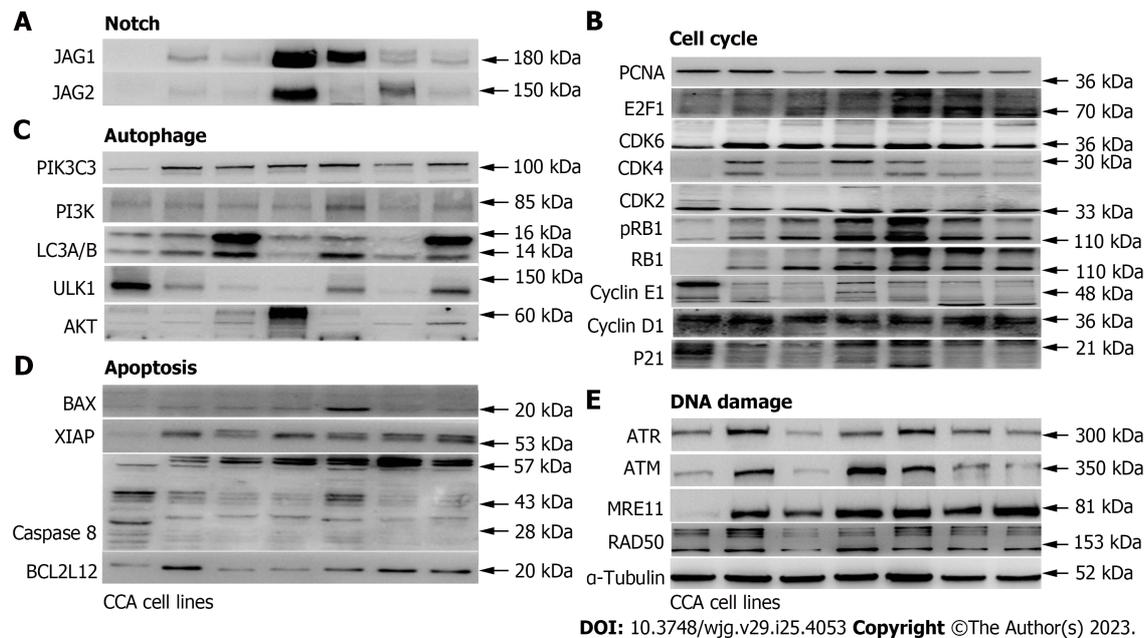


Figure 3 The cell cycle and Notch pathway associated protein expression levels are up-regulated in cholangiocarcinoma cell lines compared to the normal human bile duct cells. The protein expression levels of leading-edge genes from (A) Notch, (B) cell cycle, (C) autophagy, (D) cell death, and (E) DNA damage were determined in cholangiocarcinoma (CCA) cell lines compared to hBD. Several cell cycle and Notch associated genes, including CDK4, CDK6, E2F1, JAG1, and JAG2, were up-regulated in CCA cell lines. CCA tumor cell lines including HuCCT1, ETK-1, H1, RBE, TFK-1 and SSP25. α -Tubulin served as the loading control.

tumor were showed (Figure 4C). When comparing with human non-malignant bile duct and CCA tumor samples, the mouse CCA tumors present almost identical CCA histology to human CCA tumor samples (Figure 4C and D). To further determine whether mouse CCA and human CCA tumors have similar molecular profiles, we examined the genes that we identified using public human CCA dataset and validated using normal biliary epithelial cells and human CCA cell lines. Intriguingly, mouse and human CCA both have highly elevated genes associated with cell cycles and Notch, including CDK2, CDK4, CDK6, JAG1, and JAG2 (Figure 4E and Supplementary Figure 3). In line with CCA cell line data, several DNA damage genes, including RAD50 and MRE11, were found increased in mouse CCA tumor samples as well (Figure 4E). Given all these facts, our data demonstrated that the CCA tumors derived from the CCA transgenic mouse model have very high similarity to human CCA tissues and cell lines. Besides, the results of CCA transgenic mouse tumor data further demonstrated that refining the public human RNA sequencing data would help identify dysregulated pathways which may be druggable.

Targeting cell cycle and Notch pathways inhibited cell growth of human CCA cell lines

As we have found that cell cycle and Notch associated pathways are highly elevated in human CCA tumor samples and validated these findings in CCA cell lines, we proposed to determine whether targeting these pathways using small molecular inhibitors could achieve therapeutic potential in CCA cell lines, including H1, HuCCT1 (Kras and P53 mutations), and RBE (IDH1 mutation). It was found that the D1-dependent CDK4 inhibitor Arcyriaflavin a significantly suppressed cell growth in HuCCT1 and RBE CCA cell lines, but to a less extent in H1 (Figure 5A). The pan-CDK inhibitor Flavopiridol and the selected CDK inhibitor Roscovitine, which targets multiple CDKs, both displayed substantial growth inhibition in H1, HuCCT1, and RBE CCA cell lines (Figure 5B and C). It was noted that RBE CCA cells with IDH1 mutation are very vulnerable upon the treatments of CDK inhibitors (Figure 5A-C). Regarding the treatment using a Notch inhibitor, a γ -secretase inhibitor-DAPT was used to challenge these CCA cell lines. Although the Notch inhibitor could exert anti-tumor effects in CCA cell lines, the suppressive effects are not as strong as CDK inhibitors. Furthermore, the close relationships between the levels of TILs and those genes expressed indicated that cell cycle related pathways are associated with immune check point therapies (Supplementary Figure 4). Taken together, our results suggested that targeting cell cycle and/or Notch pathways could be considered as alternative modalities for patients with CCA.

Validation of identified cell cycle and Notch related pathways in other datasets

It is essential to verify whether our findings are applicable to other datasets. Thus, we utilized another dataset (GSE107943) to validate the oncogenic traits of cell cycle and Notch related pathways in CCA. Consistently, the leading edge of GSEA showed pathways pertaining to cell cycle (Figure 6A, upper) and Notch (Figure 6A, lower) were significantly activated in tumors compared with the normal

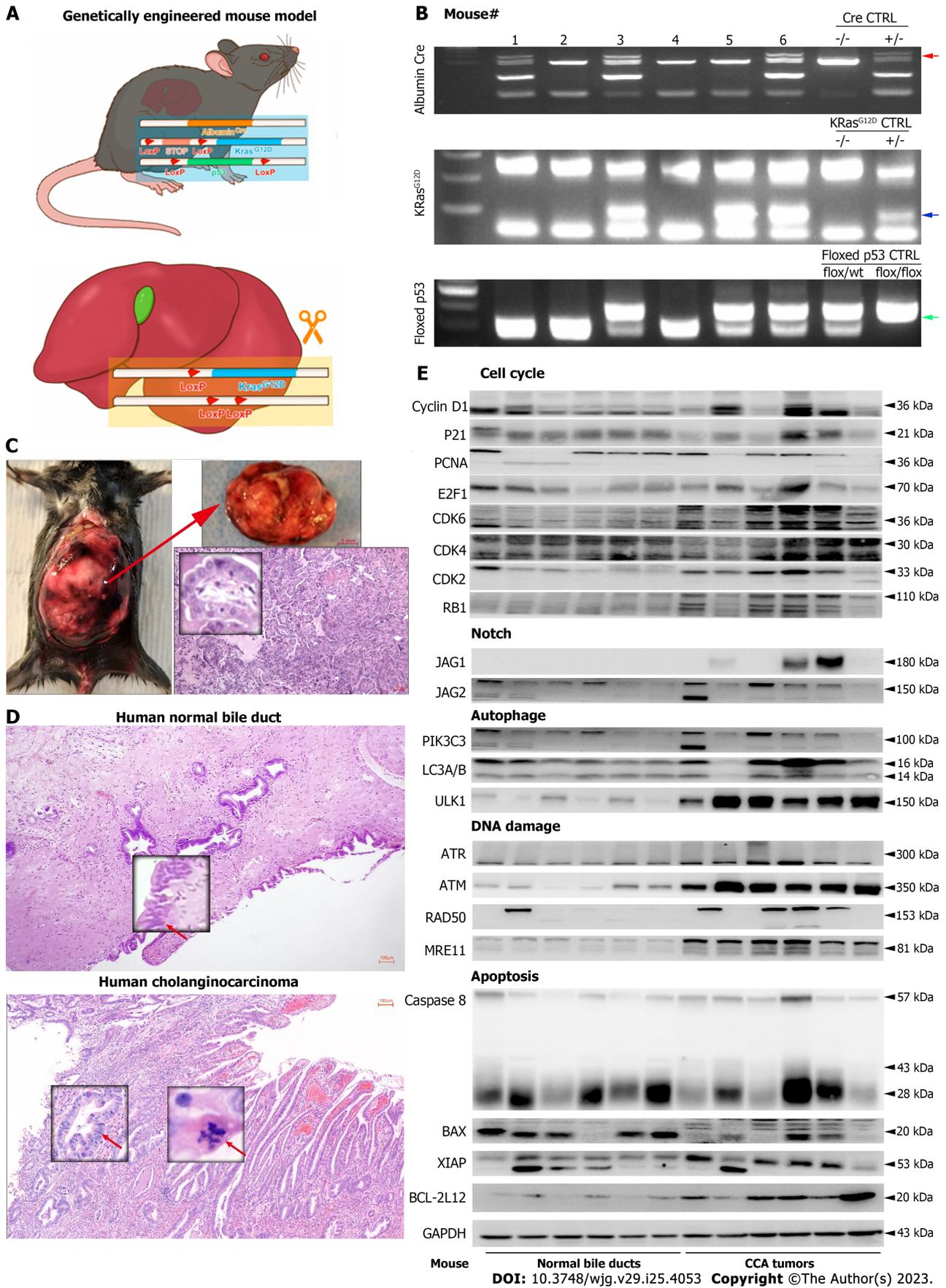


Figure 4 Validation of the identified cell cycle and Notch associated genes in the triple transgenic cholangiocarcinoma mouse model. A: The cartoon diagram of the genetically engineered mouse modeling (GEM) strategies. Triple transgenic mice bearing Albumin-Cre transgene, LSL-Kras^{G12D}, and floxed-p53 were conditionally activated Kras^{G12D} and heterozygous knocked out P53 in the liver. **B:** The genotyping data presenting positive Cre (red triangle),

KrasG12D (blue triangle), and homozygous floxed P53 genes (green triangle). C: The gross image of representative tumor (upper right, scale bar = 5 mm), and the corresponding histopathology (lower right, scale bar = 100 μ m) with inset showing glandular appearance (arrow, scale bar = 20 μ m). D: Histology of human bile duct (upper, scale bar = 100 μ m) with inset showing biliary epithelium (arrow, scale bar = 20 μ m), and cholangiocarcinoma (lower, scale bar = 100 μ m) with inset showing mitotic figure (arrowhead, scale bar = 20 μ m) and glandular appearance (arrow, scale bar = 20 μ m). E: The protein expression levels of leading-edge genes from cell cycle and Notch associated pathways, and genes involved in autophagy, cell death and DNA damage in CCA tumor tissues from the GEM (50 wk) compared to the normal bile ducts of control mice (50 wk). GAPDH was used as the loading control.

samples. Besides, the analysis of differentially expressed genes also revealed that upregulation of cell cycle related genes, including *CDK2*, *CDK4*, *CCNE1*, and *E2F1*, were observed in human CCA tumor samples compared with the adjacent normal liver tissues. Similarly, several Notch associated genes, including *NOTCH1* and *JAG2*, were found up-regulated in CCA tumor samples as well (Figure 6C). Together, with the validation data derived from another dataset, it is therefore suggested that our findings are applicable to other clinical human CCA tumor samples.

Cell cycle and Notch associated genes were correlated with poor prognosis in CCA patients

The cell cycle and Notch pathways were refined from the analysis of GSEA. These genes were found up-regulated in CCA tumor samples as well when compared with adjacent normal liver tissues. As we have found that targeting these pathways significantly suppressed CCA cell growth *in vitro*, we speculated that high expression of these genes may correlate with poor prognosis. To determine the correlation of these genes and CCA patient prognosis, we conducted the Kaplan-Meier survival analysis using the cell cycle and Notch pathways associated genes in CCA patients derived from two datasets, including GSE107943 and EBI (EGAD00001001076). It was found that high expression of cell cycle genes, including *CDK2*, *CDK6*, *CCNE1*, and *E2F1*, were negatively correlated with OS of CCA patients derived from the EBI database (EGAD00001001076) (Figure 7A). Similarly, high expression of Notch related genes, including *NOTCH1* and *JAG2*, were associated with poor prognosis in these patients (Figure 7B). These findings were further validated using the GSE107943 database. Consistently, high *CDK2*, *CDK4*, *CCNE1*, and *E2F1* were correlated with poor OS of CCA patients (Figure 7C). High *NOTCH1* expression was associated with poor OS and DFS in these patients as well (Figure 7D). Collectively, these data demonstrated that refining the signaling pathways associated with tumorigenesis of human CCA using bioinformatics approach could identify potential therapeutic targets for patients with this deadly disease (Figure 8).

DISCUSSION

The current study demonstrated that cell cycle and notch associated pathways are elevated in human CCA patients compared with adjacent normal tissues. These findings were verified using human biliary epithelial cells and CCA cell lines. Consistently, cell cycle and Notch associated genes were found increased in CCA cell lines compared with non-malignant human biliary epithelial cells. As it has not been investigated if the cell cycle and Notch associated pathways are up-regulated in the CCA transgenic mice, our studies therefore disclose the similar but novel findings in the transgenic CCA mouse model. Cell cycle and Notch associated genes were up-regulated in mouse CCA tumors when compared with age-matched mouse normal bile ducts. Besides, we demonstrated that targeting cell cycle and Notch pathways could inhibit cell growth in human CCA cell lines. Furthermore, we validated the GSEA findings using another dataset. Most importantly, we demonstrated that high expression of several cell cycle and Notch associated genes are highly associated with poor prognosis in human CCA patients in two databases.

Refining RNA sequencing results have been well studied in liver malignancy, including hepatocellular carcinoma and CCA[3,4,24,25]. By combining whole genomic sequencing and RNA sequencing data, it was previously suggested that intrahepatic CCA could be classified as inflammation and proliferation groups which have different clinical outcomes, specifically worse prognosis in the proliferation group[26]. Recently, it was also suggested that extrahepatic CCA could be classified as immune, mesenchymal, metabolic, and proliferation groups. Further analysis revealed specific potential therapeutic targets correspondent to different groups, *i.e.*, PD1/PD-L1 inhibitors were suggested to be potential therapies for the immune group of extrahepatic CCA patients[4]. Nevertheless, these potential therapeutic approaches have not been evaluated in preclinical models due to the possible reasons that no preclinical models could represent the individual group specifically. However, the recent study investigated the microenvironment-based classification of intrahepatic CCA and identified the inflammatory stroma class of intrahepatic CCA with T cell exhaustion and KRas mutation. Thus, they evaluated if the combination of KRas inhibitor and PD1 antibody could inhibit the malignant progression of CCA tumors established by using tail vein hydrodynamic injection of SB13 transposase, CRISPR/Cas9-sg-p19, pCaggs-KrasG12D. The model has high similarity to the inflammatory stroma intrahepatic CCA group. Their findings demonstrated that Kras inhibitor and PD1 antibody could

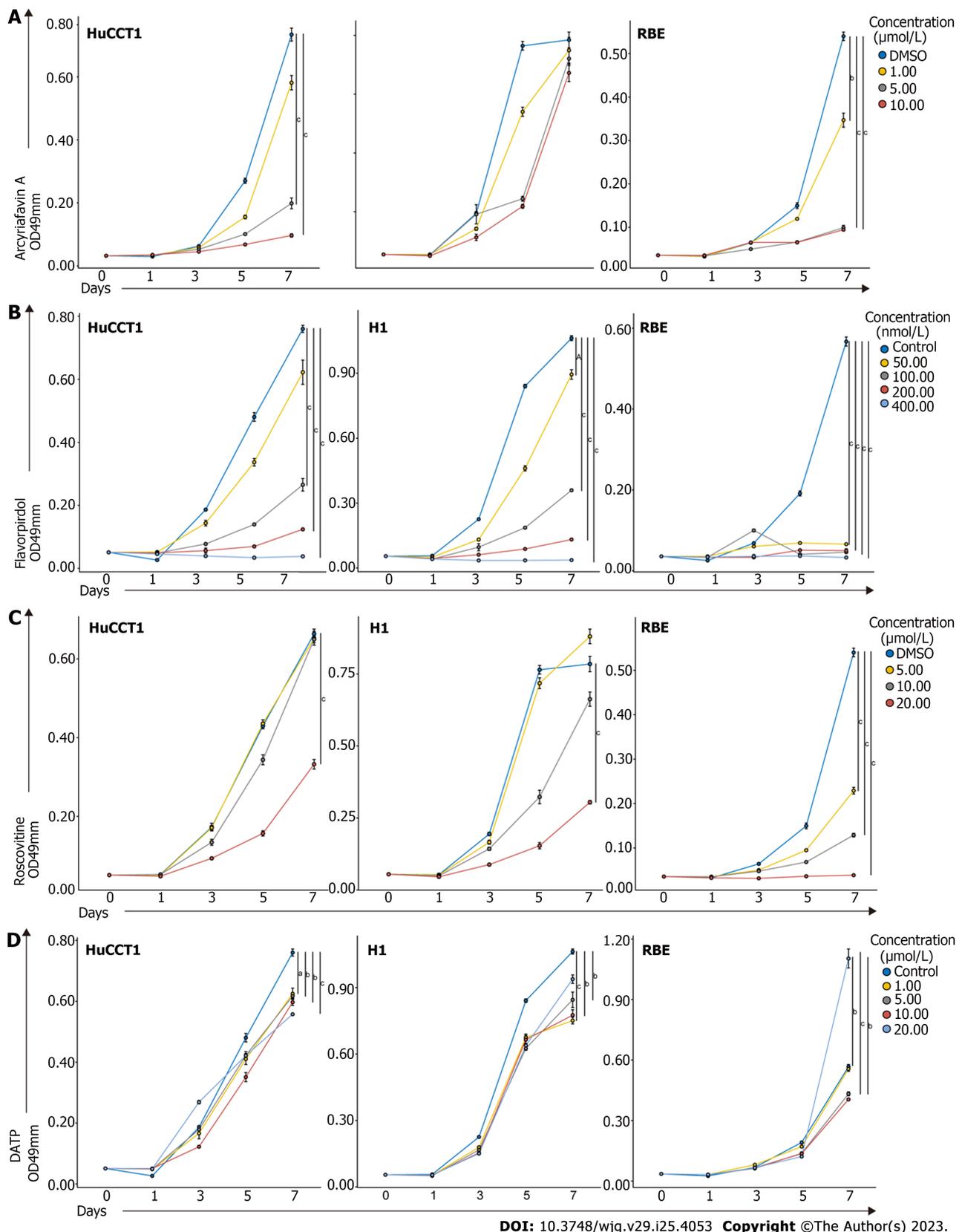
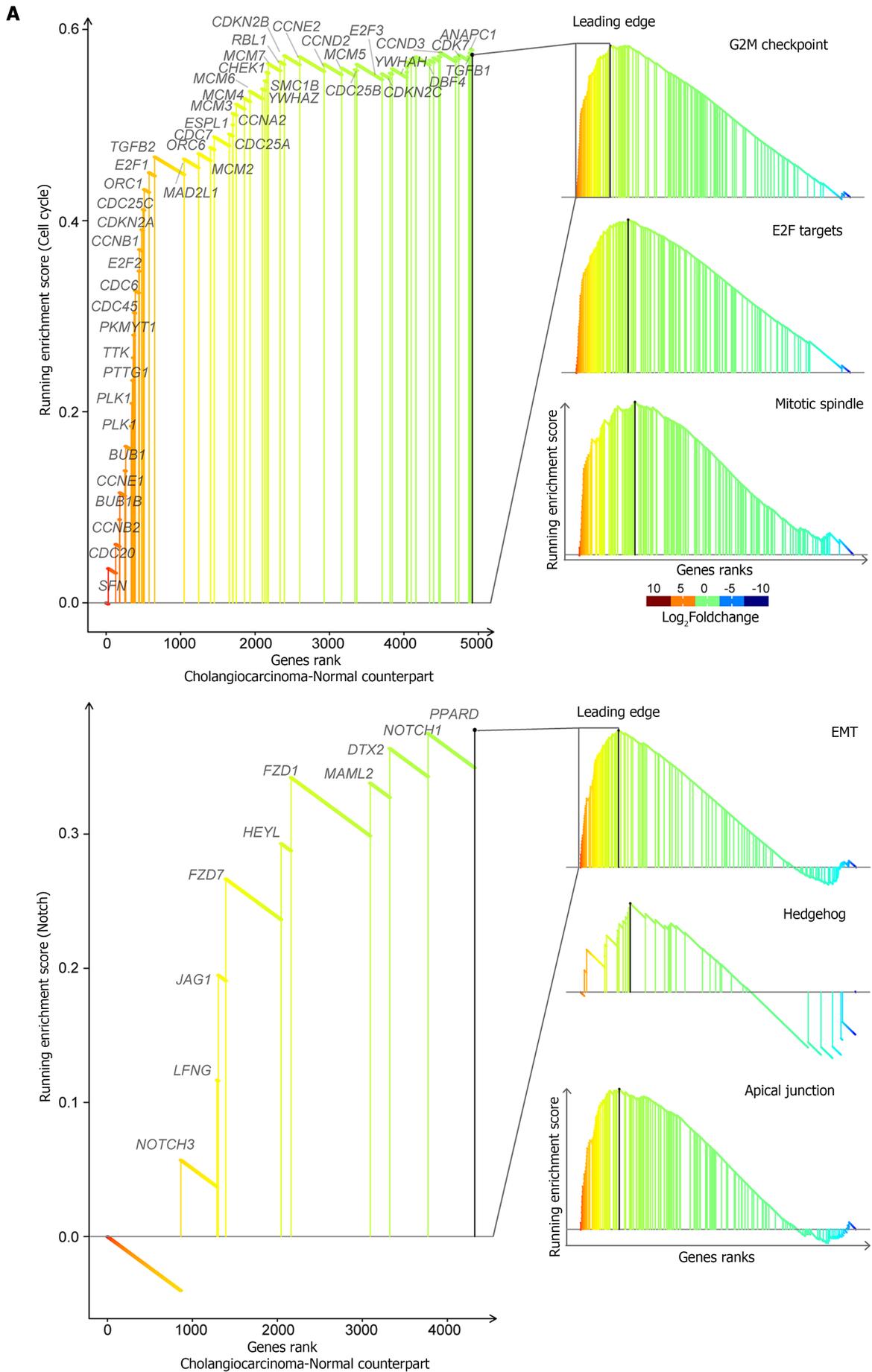


Figure 5 Targeting cell cycle and Notch pathways inhibited cell growth of human cholangiocarcinoma cell lines. Growth curves of three cholangiocarcinoma cell lines with different genetic background, e.g., HuCCT1 (KRAS and P53 mutations), RBE (IDH1 mutation), and H1, treated with cell cycle and Notch inhibitors, respectively. For targeting cell cycle pathways, (A) D1-dependent CDK4 inhibitor Arcyriaflavin a, (B) pan-CDK inhibitor Flavopiridol, (C) selected CDK inhibitor Roscovitine were utilized, and (D) the γ -secretase inhibitor (DAPT) was used against Notch pathway. Absorbance at OD570 nm and 650 nm analyzed by MTT, and then normalized to vehicle control (0.1% DMSO or the corresponding medium; $n = 6$ wells/dose point). The relative cell proliferation rates were calculated as OD570 - OD650. ^a $P < 0.05$, ^b $P < 0.01$, and ^c $P < 0.001$ when compared with the vehicle control at day 7.



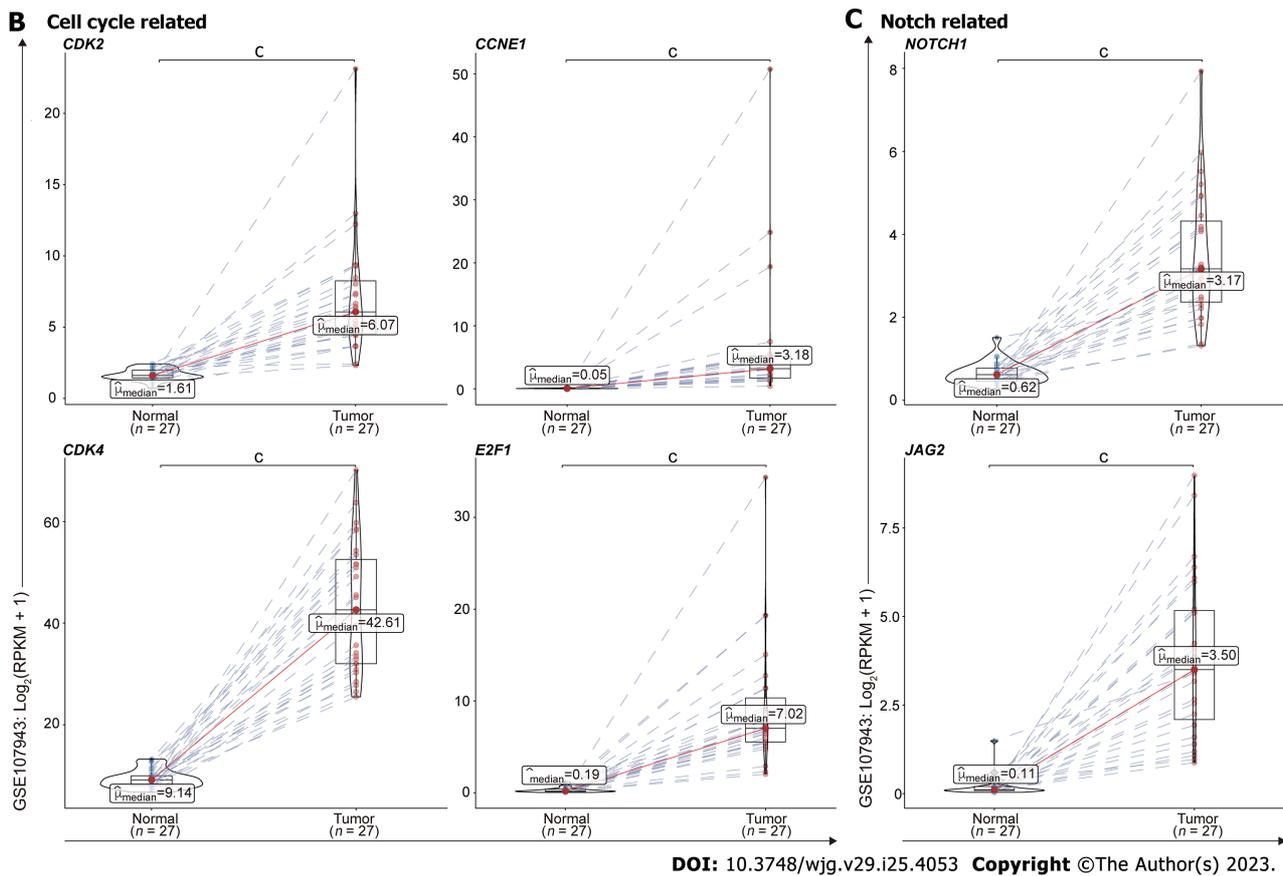
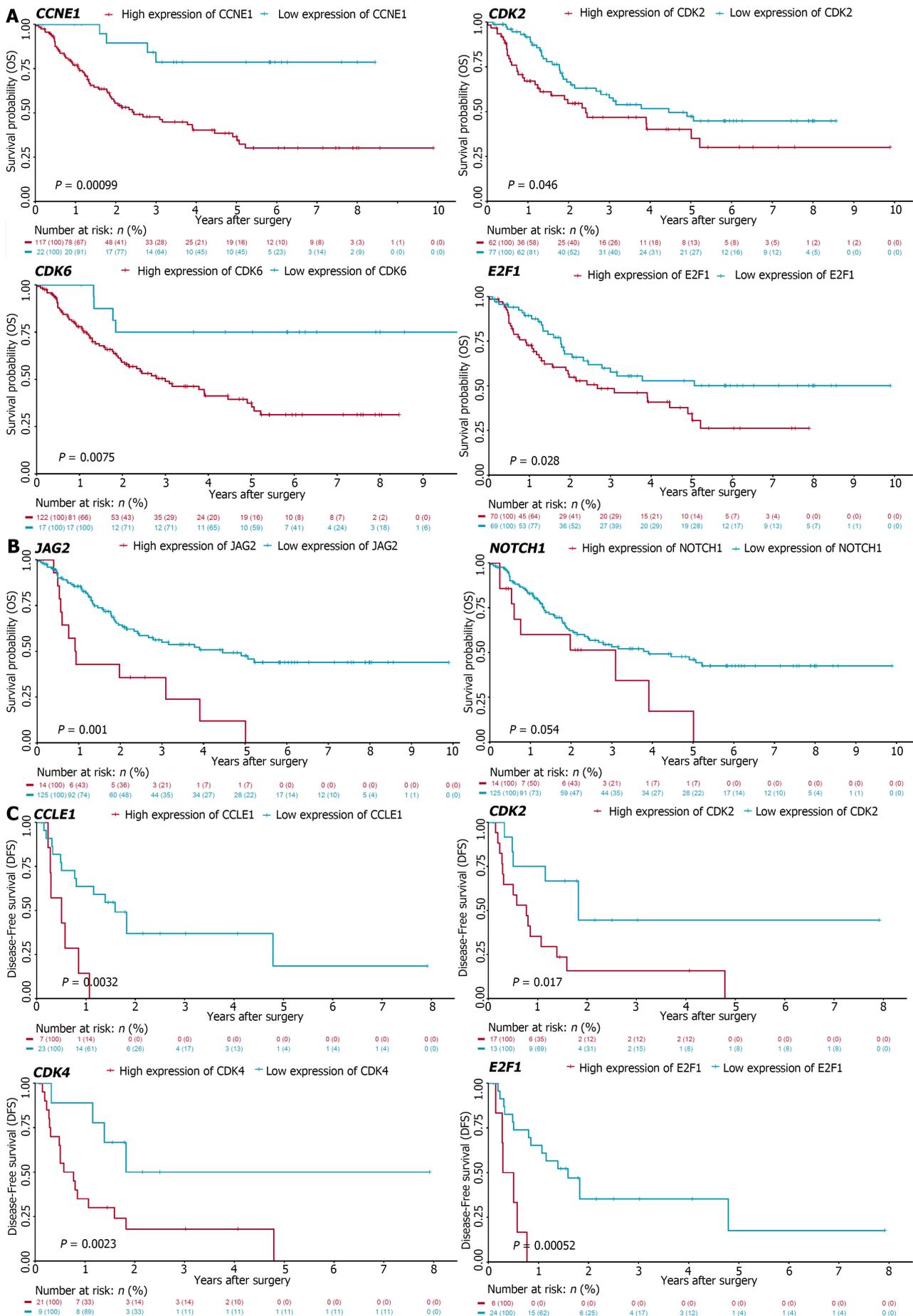


Figure 6 The generalizable oncogenic traits of cell cycle and Notch associated pathways in cholangiocarcinoma. GSEA identified cell cycle and Notch associated pathways significantly activated in tumors compared to the corresponding adjacent normal livers from GEO database (GSE107943) (A). Upper: Cell cycle associated pathways, including G2M checkpoint, E2F Targets, and Mitotic spindle gene sets; lower: Notch associated pathways, including epithelial mesenchymal transition, Hedgehog, and apical junction gene sets. The mRNA expression levels from the leading edges of cell cycle (B) and Notch (C) associated pathways were analyzed in CCA samples compared to the corresponding adjacent normal livers (pairs = 27). RPKM: Reads per kilobase million; Red: Intrahepatic cholangiocarcinoma; Blue: Para-cancerous normal liver; $^{\circ}P < 0.001$ when compared with the relevant normal control.

substantially inhibit tumor malignant progression[3]. Taken together, it is very likely to identify potential therapeutic targets by refining publicly available RNA sequencing datasets.

By using this approach, we found that Notch and cell cycle associated pathways are potential therapeutic targets in CCA tumors. It has been previously demonstrated that specific overexpression of intracellular domain of Notch 1 could lead to CCA development possibly through cyclin E1[27]. The Notch1 Ligand, JAG1 could activate Notch1 signaling cascade to initiate cyclin D1 transcriptional expression[28]. Both JAG1 and Notch1 contain the epidermal growth factor like domain[29] which could be hydroxylated by aspartate beta-hydroxylase[30-32]. Indeed, previous studies have demonstrated that aspartate beta-hydroxylase could promote CCA progression through the Notch1-mediated cyclin D1 pathway[33]. Cell cycle is tightly controlled by the tumor suppressor retinoblastoma protein (RB1)[34]. Although RB1 mutation is not a common event in CCA patients[35], dysregulated cell cycle regulating genes are often found in these patients. Dysregulated cell cycle controlling genes are often found in these patients. Dysregulated cell cycle controlling genes would lead to cancer cell proliferation. As discussed, intrahepatic CCA patients could be classified as proliferative and inflammatory groups[26]. Previous studies also demonstrated the therapeutic potential of targeting CCA tumors by using the specific inhibitor targeting cyclin dependent kinases 4 and 6[36,37] which are important mediator controlling RB1 phosphorylation. Phosphorylation of RB1 prevents its binding from the E2F1 transcriptional factor, which finely modulates cell cycle regulating genes[38]. Thus, phosphorylation of RB1 would lead to inactivation of RB1 tumor suppressor function, in turn promoting cell cycle progression. It was previously reported that aspartate beta-hydroxylase could promote RB1 phosphorylation to disrupt cell cycle regulation in CCA tumors[39]. Interestingly, aspartate beta-hydroxylase was reported to be highly expressed in CCA but barely detectable in normal bile duct tissues[39], suggesting the linkage of identified Notch1 and cell cycle associated pathways to aspartate beta-hydroxylase.

The current study has several limitations. (1) We did not evaluate if targeting Notch1 and cell cycle pathways could inhibit CCA progression using preclinical models. Actually, the previous studies have demonstrated that Notch1 inhibitor could be a potential therapeutic approach[27] and that targeting cell cycle with CDK4/6 inhibitors could suppress CCA progression[36,37]. Although the current study was



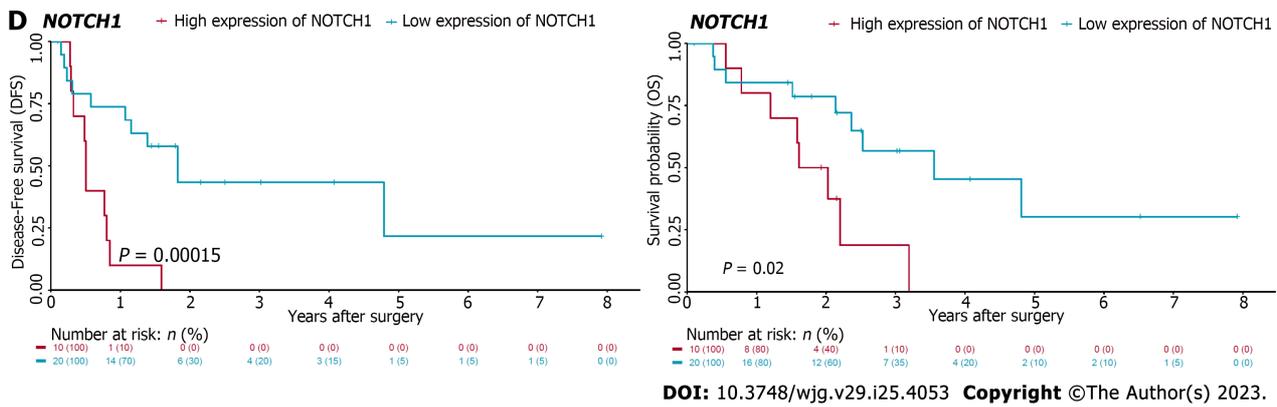


Figure 7 Cell cycle and Notch associated genes correlate with dismal survival in patients with cholangiocarcinoma. Kaplan-Meier analysis of cholangiocarcinoma patients in EBI-EGAD00001001076 (A and B) and GSE107943 (C and D) database. The optimal cutoff of genes from the leading edges of cell cycle (A and C) and Notch (B and D) associated pathways was calculated by maximally selected rank statistics. And then the difference between two groups was analyzed by log-rank test. Red: Higher expression of the indicated genes; Blue: Lower expression of indicated genes; DFS: Disease-free survival, OS: Overall survival.

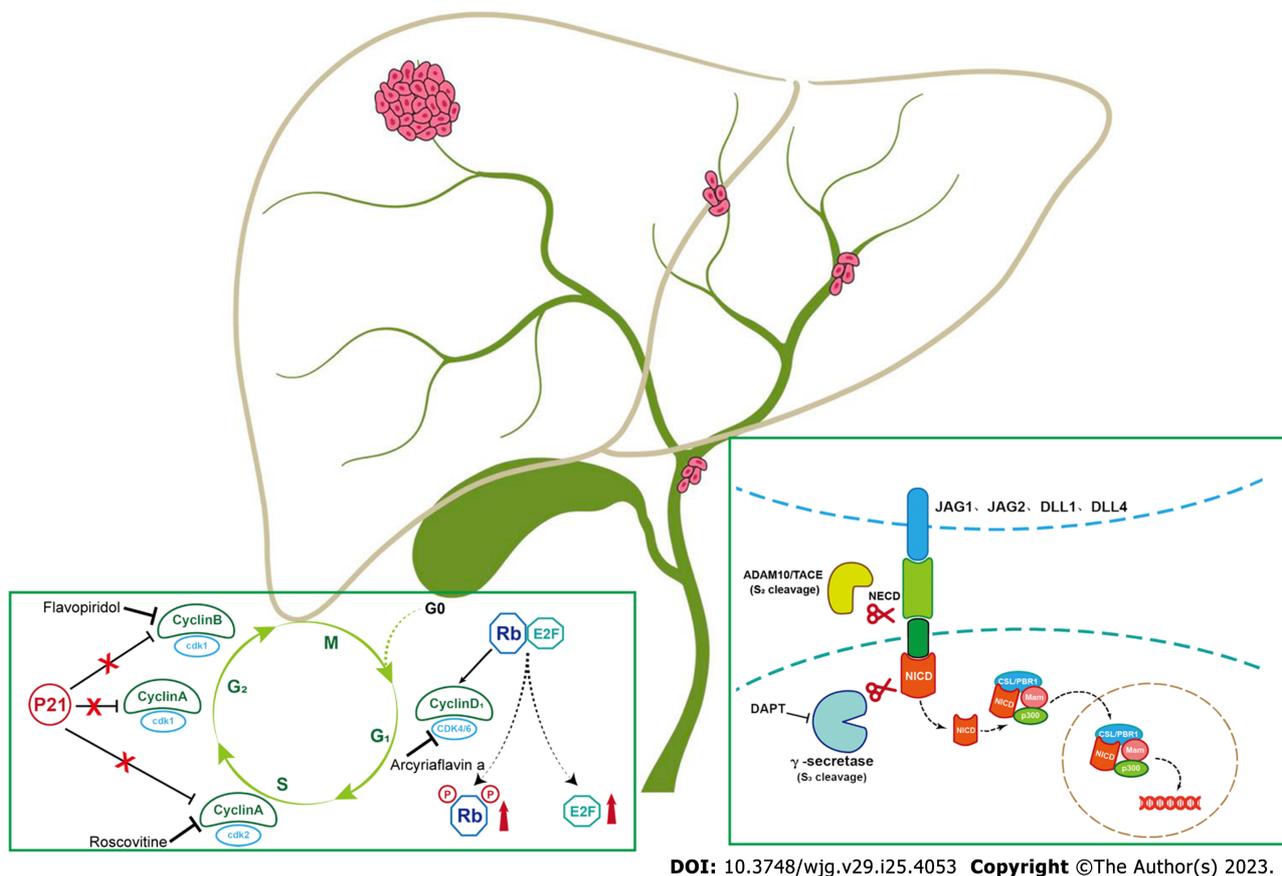


Figure 8 The summary of dysregulated cell cycle and Notch pathways in cholangiocarcinoma progression. The cholangiocarcinogenesis driven by aberrant cell cycle and Notch pathways, which may be targeted by small molecular inhibitors.

initiated from a different angle by using transcriptomic analysis, it ended up with similar findings that Notch1 and cell cycle pathways could be potential targets in CCA patients; (2) We did not separate intrahepatic and extrahepatic CCA patients. We pooled the available data by combining intrahepatic and extrahepatic transcriptomic datasets due to the limited datasets publicly available; and (3) our studies did not investigate if elevation of cell cycle and Notch associated pathways may be used as early detection markers. Future studies will be needed to further determine potential therapeutic approaches specifically for intrahepatic and extrahepatic CCA patients, respectively.

CONCLUSION

In conclusion, our data, through mRNA and protein profiling, identified very high similarity between CCA tumors derived from the CCA transgenic mice and human CCA tumors, thus providing a potential preclinical CCA model for investigating the CCA tumorigenesis. Besides, our data suggested that cell cycle and Notch associated pathways could be potential therapeutic targets in CCA patients.

ARTICLE HIGHLIGHTS

Research background

The molecular pathogenesis of cholangiocarcinoma (CCA) remains largely unknown. Investigating the molecular mechanisms underlying CCA progression will potentially yield results toward identifying therapeutic targets.

Research motivation

Several mRNA sequencing (Seq) datasets in CCA samples are publicly available, but it remains unclear if the data can be validated in CCA cell lines, the transgenic CCA mouse model, and human CCA samples.

Research objectives

To summarize the mRNA Seq results and validate the findings in CCA cell lines, the transgenic CCA mouse model, and human CCA samples.

Research methods

Bioinformatic analysis, cell culture studies, transgenic mouse model, human CCA samples, and molecular strategies were used to determine molecular pathogenesis of CCA.

Research results

Through bioinformatic analysis, we found that cell cycle and Notch associated pathways are up-regulated in human CCA samples, compared to the non-tumor controls. We validated the findings in human CCA cell lines and the transgenic CCA mouse model.

Research conclusions

Our data, through mRNA and protein profiling, identified very high similarity between CCA tumors derived from the CCA transgenic mice and human CCA tumors, thus providing a potential preclinical CCA model for investigating CCA tumorigenesis. Besides, our data suggested that cell cycle and Notch associated pathways could be potential therapeutic targets in CCA patients.

Research perspectives

Systemic chemotherapy, gemcitabine and cisplatin, is recommended for CCA patients. However, the 5-year survival of CCA patients has not been significantly improved, suggesting an urgent need to develop novel therapeutic approaches.

FOOTNOTES

Author contributions: Huang CK contributed to the study concept and design; Liu D, Shi Y, Chen H, Nisar MA, Jabara N, Langwinski N, Mattson S, Nagaoka K, Bai X and Huang CK contributed to the acquisition of data; Liu D, Shi Y, Bai X, Huang CK and Lu S contributed to analysis and interpretation of data; Liu D, Lu S, Jabara N, Nagaoka K, Chen H and Nisar MA contributed to the technical support; Lu S contributed to the material support; Huang CK and Lu S obtained the funding; Liu D, Shi Y, Huang CK, Lu S and Nisar MA drafted the manuscript; Shi Y, Nisar MA, Huang CK, Lu S, Jabara N, Langwinski N and Mattson S revised the manuscript. Liu D and Shi Y contributed equally.

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