

The cyclin-dependent kinase pathway involving CDK1 is a potential therapeutic target for cholangiocarcinoma

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Abstract. Cholangiocarcinoma is an aggressive malignancy with high mortality, and effective therapeutic agents for this cancer are limited. Cyclin-dependent kinase (CDK) pathways are therapeutic targets for various types of cancers; however, their involvement in cholangiocarcinoma remains unclear. The present study examined the biological significance of CDK pathways in cholangiocarcinoma. An immunohistochemical analysis of cholangiocarcinoma tissue sections revealed the upregulated expression of phosphorylated cyclin-dependent kinase 1 (p-CDK1), p-CDK2, cyclin B1, and cyclin E1 in carcinoma cells. The nuclear expression of p-CDK1 and cyclin B1 was positively correlated with the presence of lymph node metastasis and the clinical stage, and p-CDK1 expression was also associated with poor patient survival. The treatment of human cholangiocarcinoma cell lines (CCKS-1, TFK-1 and HUCCT-1) with the multi-CDK inhibitor roscovitine decreased p-CDK1 expression, inhibited cell proliferation, arrested the cell cycle at the G1 or G2/M phase, and significantly inhibited carcinoma cell invasion. *In vivo* studies using a murine xenograft model revealed that an intraperitoneal injection of roscovitine significantly inhibited cholangiocarcinoma cell growth. Roscovitine induced apoptosis in cholangiocarcinoma cells *in vitro* and *in vivo*. These results demonstrated the potential of the CDK pathway involving CDK1 as a therapeutic target for cholangiocarcinoma. Furthermore, the immunohistochemical expression of p-CDK1 may be a useful prognostic marker of cholangiocarcinoma.

Introduction

Cholangiocarcinoma is an aggressive malignancy, and the difficulties associated with its diagnosis at the operative stage and high recurrence rate after surgery result in high mortality (1,2). Cholangiocarcinoma may be resistant to conventional chemotherapeutic agents, and non-surgical therapeutic regimens result in minimally improved patient survival (3). Therefore, the discovery of new therapeutic targets is required to increase favorable outcomes among cholangiocarcinoma patients.

Cyclin-dependent kinases (CDKs) belong to a family of protein kinases that play critical roles in the regulation of the cell cycle machinery, and consist of 21 hypotypes (4). CDK activation is required for cell cycle progression, and is positively regulated by cyclins and negatively regulated by CDK inhibitors. CDK1-cyclin B was revealed to be important for the progression of G2/M, and CDK2-cyclin E promoted cell cycle transition from the G1 to S phase. Furthermore, CDK5 has been revealed to be associated with the regulation of cell migration and invasion (4).

The cell cycle is dysregulated during the carcinogenic process, which is accompanied by the overexpression of positive cell cycle regulators (CDKs and cyclins) and the loss of function of CDK inhibitors (5-7). Dysregulated CDKs have been linked to cancer initiation and progression, and the up-regulated expression of CDKs is closely associated with the poor prognosis of patients with various types of cancers (8-12). Thus, the targeting of CDK pathways represents an effective therapeutic strategy against cancer, and there are several ongoing clinical trials using CDK inhibitors (13).

In pancreatic ductal adenocarcinoma (PDAC), the pharmacological inhibition of CDKs was revealed to be effective in experimental studies, and several CDK inhibitors are currently being examined in clinical trials (13-15). A recent study reported that CDK1 is a synthetic lethal target for KRAS mutant tumors including PDAC (16). Since activating KRAS mutations have been reported in cholangiocarcinoma (17-19), and PDAC shares many biological properties with cholangiocarcinoma, the inhibition of CDKs may be beneficial for the treatment of cholangiocarcinoma.

Several experimental studies on cholangiocarcinoma have examined alterations in the expression of CDKs and cell cycle progression following treatments with anticancer agents other than CDK inhibitors (20-23). However, evidence to directly support the involvement and biological significance of the CDK

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Abbreviations: CDK, cyclin-dependent kinase; DAB, 3,3'-diaminobenzidine tetrahydrochloride; ELISA, enzyme-linked immunosorbent assay; p-CDK, phosphorylated CDK; PDAC, pancreatic ductal adenocarcinoma; SD, standard deviation; TUNEL, terminal dUTP nick-end labeling

Key words: cholangiocarcinoma, cyclin-dependent kinase, cell cycle, chemotherapy, prognosis

pathways in cholangiocarcinoma is limited. Therefore, we herein examined CDK pathways in cholangiocarcinoma, with the aim of developing a novel therapeutic approach for the disease.

Materials and methods

Ethical statement. Experiments using human materials were performed with the approval of the Ethics Committee of Kanazawa University Graduate School of Medicine (Permit no. 1985-3). Protocols for animal studies were approved by the Committee of the Institute for Experimental Animals, Kanazawa University Advanced Science Research Center (Permit no. AP-173905).

Cell culture. The human cholangiocarcinoma cell lines, CCKS-1, TFK-1 and HUCCT-1, were used. CCKS-1 was established in our laboratory from abdominal metastasis of cholangiocarcinoma (moderately differentiated adenocarcinoma) (24). TFK1 and HUCCT-1 were provided by Cell Resource Center for Biochemical Research, Tohoku University, Sendai, Japan. CCKS-1 was grown in Dulbecco's modified Eagle's medium/F-12 (Gibco; Thermo Fisher Scientific, Inc.), and TFK-1 and HUCCT-1 were grown in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum and 1% antibiotic-antimycotic solution (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in an atmosphere of 5% CO₂. To block CDK signaling, the multi-CDK inhibitor roscovitine (Cell Signaling Technology, Inc.) was used.

Cell proliferation assay. Cell proliferative activity was examined using the WST-1 assay (Roche Diagnostics GmbH) according to the manufacturer's instructions. Cells were seeded on a 96-well plate at a density of $\sim 2 \times 10^3$ – 1×10^4 cells/well. Cells were treated with roscovitine at the concentrations of 2, 10 and 20 μ M. After 24, 48 and 72 h following the treatment, the WST-1 reagent was added and incubated for 1 h before reading the plate. Absorbance at 450 nm was measured using a microplate reader. Each assay was conducted in eight sets.

Flow cytometry. Cells were treated with 20 μ M roscovitine for 24 h and processed for cell cycle analysis using the BrdU Flow Kit (Nippon Becton Dickinson Company, Ltd.) according to the manufacturer's instructions. Samples were analyzed using BD FACSCanto™ II Flow Cytometry (BD Biosciences). The percentages of cells in the G1, S, and G2/M phases of the cell cycle were assessed using BD FACSDiva™ Software (BD Biosciences).

Western blotting. Cells were treated with 20 μ M roscovitine for 72 h, and proteins were extracted using the T-PER Protein Extraction Reagent (Gibco; Thermo Fisher Scientific, Inc.). Protein concentrations were determined using the Bradford method. Equal amounts of protein (50 μ g) were loaded in each lane of an SDS-polyacrylamide gel (5–20%). Proteins were separated by electrophoresis and transferred to a nitrocellulose membrane. After blocking, the membrane was incubated at 4°C overnight with primary antibodies against p-CDK1 (Thr161; 1:500 dilution; cat. no. bs-3481R; rabbit polyclonal; Bioss Antibodies, Inc.), CDK1 (1:1,000 dilution; mouse monoclonal; cat. no. ab18; Abcam), and β -actin (1:1,000 dilution;

cat. no. 4967). rabbit monoclonal; Cell Signaling Technology, Inc.). Following an incubation with the secondary antibody conjugated with peroxidase using Histofine Simple Stain MAX PO (Nichirei Corp.; cat. no. 424142) at room temperature for 1 h, the protein was visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB). Image analysis was performed using the NIH ImageJ software (National Institutes of Health). Each assay was conducted in five sets.

ELISA assay. The protein levels of p-CDK1 (Thr161) and total CDK1 in cholangiocarcinoma cell lines were determined using an enzyme-linked immunosorbent assay (ELISA). The same samples used for the western blotting were analyzed, and the protein levels were determined using a RayBio® Human phospho-CDK1 (Thr161) and Total CDK1 ELISA kit (RayBiotech) according to the manufacturer's instructions. Optical density was measured using a microplate reader at 450 nm, and the ratio of p-CDK1/CDK1 was calculated for each sample. Each assay was conducted in six sets.

Apoptosis assay. DNA fragmentation attributable to apoptosis was assessed by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method. Cells were treated with 20 μ M roscovitine for 72 h and processed in a paraffin-embedded cell block. After deparaffinization, sections were stained using a commercial kit (*in situ* Apoptosis Detection kit; Takara Bio, Inc.) according to the manufacturer's instructions. Color development was performed using DAB, and sections were counterstained with hematoxylin. Five high-power fields were randomly selected under a light microscope, and the percentage of apoptotic cells was calculated.

Invasion assay. Carcinoma cell invasion was examined using the Corning® BioCoat™ Matrigel® Invasion Chamber (Corning, Inc.) according to the manufacturer's instructions. Cells (~ 2 – 15×10^4 cells/well) in a 0.5-ml suspension with or without 20 μ M roscovitine were seeded in each well. Cells were incubated at 37°C for 72 h and then fixed at room temperature for 12 h with 10% neutral formalin. Cells were stained with Diff-Quick (Sysmex Corp.) at room temperature for 15 sec. Migrated cells were visualized under a light microscope, and the number of cells was counted in five randomly selected high-power fields.

Murine xenograft model. Five-week-old BALB/cAnNCrj-nu/nu nude mice (male) were obtained from Charles River Laboratories, Inc. CCKS-1 was implanted subcutaneously into the right flanks of mice. On day 20, tumor-bearing mice were randomly divided into two groups (control and treatment groups), and five mice were included in each group. Roscovitine (ChemScene) was administered to mice by an intraperitoneal injection at a dose of 50 mg/kg for two 5-day series with a 2-day break in between. Control mice were treated with carrier solution alone. Tumor sizes were measured every day, and the tumor volumes were calculated using the following formula: $(1/2) L \times W^2$, where L and W represented the longest tumor axis (mm) and the shortest tumor axis (mm), respectively. The fold difference from the initial tumor volume (measured at day 20) was defined as the tumor growth rate (25).

On day 33 (13 days after the first injection of roscovitin), the mice were sacrificed by inhalation of CO₂ with a 20% volume/min gas displacement flow rate until euthanasia was confirmed by the arrest of heartbeat and breathing. Formalin-fixed paraffin-embedded sections of dissected tumors were used for the apoptosis assay and the immunostaining of Ki-67.

Immunohistochemistry. Fifty-four cases of extrahepatic cholangiocarcinoma collected from Kanazawa University Hospital and its affiliated hospitals were examined. Sample collection was conducted during the periods from August 1994 to July 2016. All cases surgically resected were diagnosed as conventional cholangiocarcinoma. The median age of the patients was 69 years ranging from 35 to 84 years, and the male:female ratio was 33:21. The tumor-node-metastasis (TNM) classification was used according to the guidelines of the International Union Against Cancer (26). Cholangiocarcinoma cell lines (CCKS-1, TFK-1, and HUCCT-1) and tumor tissues obtained from a murine xenograft model were also used for the immunohistochemical analysis. Samples were fixed at room temperature for 48 h in 10% neutral formalin, embedded in paraffin, and 4-μm-thick paraffin-embedded sections were prepared.

After deparaffinization, antigen retrieval was performed by microwaving in 10 mmol/l citrate buffer (pH 6.0) for the immunostaining of p-CDK1, cyclin B1, and cyclin E1. Regarding the immunostaining of Ki-67, antigen retrieval was performed by heating in Tris-ethylenediaminetetraacetic acid buffer (pH 9.0) with a pressure cooker. Sections were immersed in 0.3% hydrogen peroxidase in methanol for 20 min to block endogenous peroxidase activity and then incubated in protein block solution (Dako; Agilent Technologies, Inc.). Sections were incubated overnight at 4°C with primary antibodies against p-CDK1 (Thr161; 1:100 dilution; cat. no. bs-3481R; rabbit polyclonal; Bioss Antibodies, Inc.), cyclin B1 (1:100 dilution; cat. no. ab32053; rabbit; monoclonal; Abcam), cyclin E1 (1:100 dilution; cat. no. ab33911; rabbit monoclonal; Abcam), or Ki-67 (prediluted; cat. no. 418071; rabbit monoclonal; Nichirei Corp.). Sections were then incubated with the secondary antibody conjugated with peroxidase using Histofine Simple Stain MAX PO [Nichirei Corp. (cat. no. 424142)] at room temperature for 1 h. Color development was performed using DAB, and sections were counterstained with hematoxylin.

The immunostaining of p-CDK2 was performed using the Novolink™ Polymer Detection System (Leica Biosystems) according to the manufacturer's protocol. Briefly, after blocking endogenous peroxidase using Peroxidase Block for 5 min and a pretreatment with Protein Block for 5 min, sections were incubated at 4°C overnight with the primary antibody against p-CDK2 (Thr160; 1:100 dilution; rabbit polyclonal; Abcam cat. no. ab194868). Sections were then treated with Post Primary Block followed by Novolink Polymer at room temperature for 30 min. Immunoreactivity was visualized using DAB Working Solution, and sections were counterstained with hematoxylin.

Histological evaluation. A semiquantitative analysis of the immunohistochemical staining of p-CDK1, p-CDK2, cyclin B1, and cyclin E1 was performed for surgically resected specimens. Staining in the nuclei or cytoplasm of

cholangiocarcinoma cells was individually evaluated, and staining in each section was graded as follows: (-), negative or <5% of carcinoma cells were positive; (+), >5% of carcinoma cells were positive.

The expression of Ki-67 was assessed by counting at least 1,000 cholangiocarcinoma cells in an area with the greatest staining density that was selected in an x200 magnification field under a light microscope. The percentage of cells that were positive for Ki-67 was expressed as the Ki-67-labeling index.

Statistical analysis. Data were expressed as the mean ± standard deviation (SD). The significance of differences was analyzed using the Student's t-test and chi-squared test. In the univariate analysis, postoperative survival probability was calculated by the Kaplan-Meier method, and survival curves were compared by the Log-rank test using BellCurve® for Excel software (Social Survey Research Information Co., Ltd.). A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Upregulation of CDK and cyclin expression in cholangiocarcinoma tissues. The immunohistochemical expression of p-CDK1, p-CDK2, cyclin B1, and cyclin E1 was absent or weak in non-neoplastic biliary epithelial cells. In cholangiocarcinoma, the upregulation of protein expression was observed in a number of cases (Fig. 1). Positive immunohistochemical signals for CDKs and cyclins were observed in both the nuclei and cytoplasm of carcinoma cells, and the extent of positive staining varied from patchy to diffuse. Among the 54 cholangiocarcinoma cases examined, positive nuclear expression for p-CDK1, p-CDK2, cyclin B1, and cyclin E1 was observed in 29 cases (54%), 18 cases (33%), 32 cases (59%), and 34 cases (63%), respectively (Table I). No correlations were noted between the positivity of p-CDK1 and cyclin B1 or the positivity of p-CDK2 and cyclin E1 (data not shown).

Clinicopathological significance of CDK and cyclin expression in cholangiocarcinoma. The relationship between clinicopathological factors and the immunohistochemical expression of CDKs and cyclins was examined. The nuclear expression of p-CDK1 and cyclin B1 was positively associated with the presence of lymph node metastasis and the clinical stage (Table I). The nuclear expression of p-CDK2 and cyclin E1 was not associated with these clinicopathological factors. The expression of p-CDK1, p-CDK2, cyclin B1, and cyclin E1 in the cytoplasm of carcinoma cells was not associated with any clinicopathological factors (data not shown).

Postoperative follow-up data were analyzed with a median follow-up period of 21.8 months for the overall survival ratio and 18.0 months for the disease-free survival ratio. Patients with the positive nuclear expression of p-CDK1 had a significantly poor prognosis (Fig. 2). In contrast, no correlation was observed between patient prognosis and the nuclear expression of p-CDK2, cyclin B1, and cyclin E1 (Fig. 2). The cytoplasmic expression of p-CDK1, p-CDK2, cyclin B1, and cyclin E1 was not associated with patient prognosis (data not shown).

A group of disease-free surviving individuals was observed within the p-CDK1-positive cohort of patients.

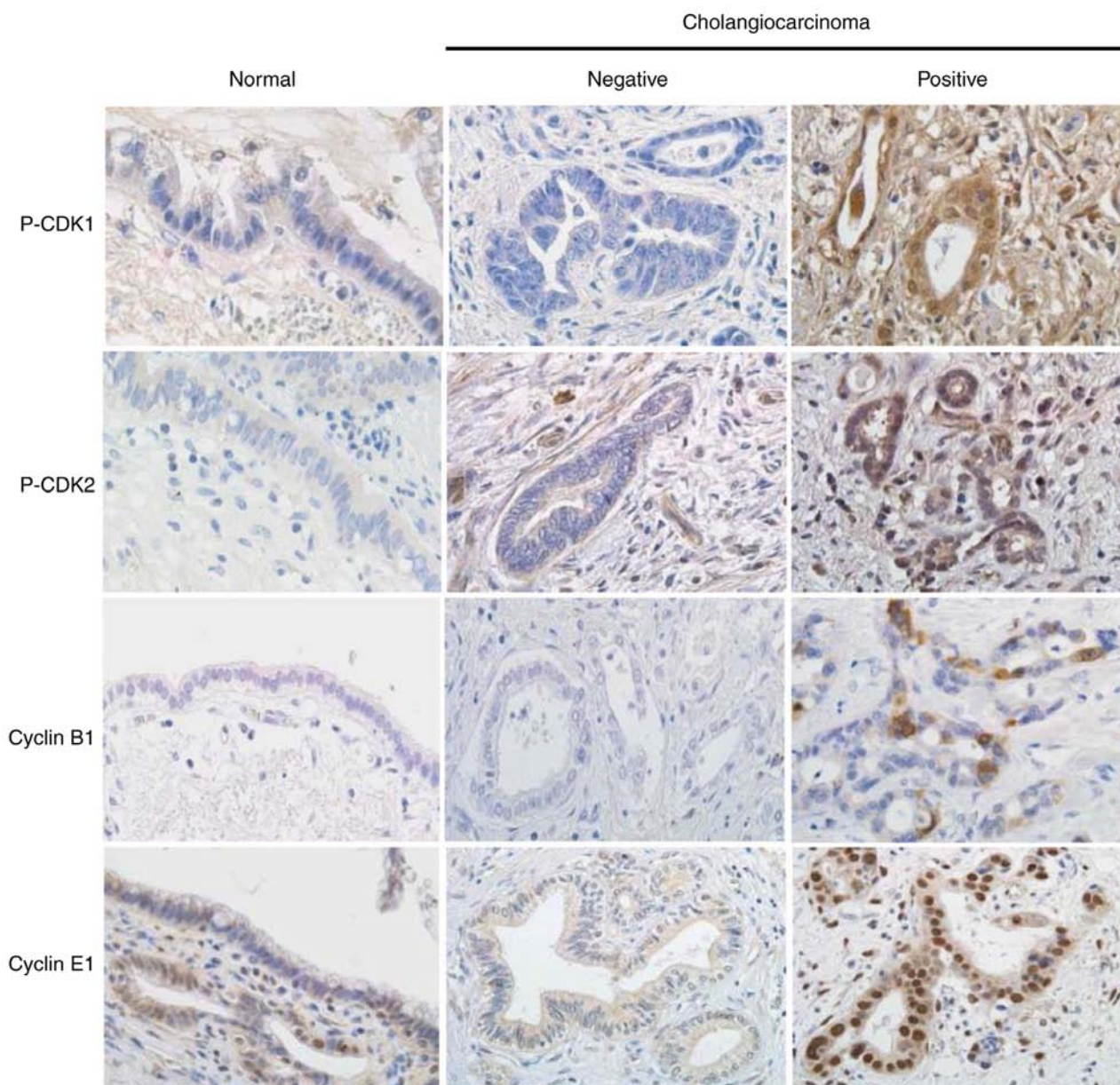


Figure 1. Immunohistochemical expression of CDKs and cyclins in cholangiocarcinoma. The expression of p-CDK1, p-CDK2, cyclin B1, and cyclin E1 was observed in a number of cholangiocarcinoma cases. The expression of these molecules in normal biliary epithelial cells was absent or weak. Original magnification, x400. CDKs, cyclin-dependent kinases; p-CDK1, phosphorylated cyclin-dependent kinase 1.

Disease-free surviving patients were also present in the p-CDK2-, cyclin B1-, and cyclin E1-negative groups. These surviving patients appeared to have cholangiocarcinoma with a well-differentiated histology.

The present results indicated that the activation of the CDK pathway involving CDK1 rather than CDK2 defined the tumor aggressiveness of cholangiocarcinoma. To further clarify their involvement in cholangiocarcinoma, *in vitro* experiments were performed.

Effects of CDK inhibition on cholangiocarcinoma biology *in vitro*. The human cholangiocarcinoma cell lines, CCKS-1, TFK-1, and HUCCT-1 were used. Immunostaining revealed that p-CDK1 was expressed in the nuclei of all three cell lines, and its expression was stronger in CCKS-1 and HUCCT-1 than in TFK-1 (Fig. 3A). When cells were treated with the

multi-CDK inhibitor roscovitine, cell proliferative activity was inhibited in the three cell lines in time- and dose-dependent manners, with 20 μ M roscovitine exerting the strongest effects (Fig. 3B). In subsequent *in vitro* experiments, roscovitine was used at a dose of 20 μ M.

The western blot analysis indicated that the treatment with roscovitine reduced the expression of p-CDK1 in the cells (Fig. 3C), and the analysis using ELISA further confirmed that the ratio of p-CDK1/CDK1 was significantly reduced by the treatment (Fig. 3D). Following treatment with roscovitine, the cell cycle was arrested at the G1 or G2/M phase in CCKS-1, TFK-1, and HUCCT-1 (Fig. 4), and this was accompanied by an increase in the percentage of apoptotic cells (Fig. 5A and B). Furthermore, the invasion of cholangiocarcinoma cells was significantly inhibited by the treatment with roscovitine (Fig. 5C and D).

Table I. Relationship between the immunohistochemical expression of CDKs/cyclins and clinicopathological factors in cholangiocarcinoma.

Factor	N (%)	p-CDK1 ^a		P-value	p-CDK2 ^a		P-value	Cyclin B1 ^a		P-value	Cyclin E1 ^a		P-value
		- (%)	+ (%)		- (%)	+ (%)		- (%)	+ (%)		- (%)	+ (%)	
Sex													
Male	33 (61)	16 (48)	17 (52)	0.686	21 (64)	12 (36)	0.554	14 (42)	19 (58)	0.752	11 (33)	22 (67)	0.480
Female	21 (39)	9 (43)	12 (57)		15 (71)	6 (29)		8 (38)	13 (62)		9 (43)	12 (57)	
Histology													
Well	26 (48)	13 (50)	13 (50)	0.599	16 (62)	10 (38)	0.441	13 (50)	13 (50)	0.182	11 (42)	15 (58)	0.440
Mod-Poor	28 (52)	12 (43)	16 (57)		20 (71)	8 (29)		9 (32)	19 (68)		9 (32)	19 (68)	
Depth of invasion													
T1, 2	28 (52)	13 (46)	15 (54)	0.984	18 (64)	10 (36)	0.070	14 (50)	14 (50)	0.151	12 (43)	16 (57)	0.358
T3, 4	26 (48)	12 (46)	14 (54)		18 (69)	8 (31)		8 (31)	18 (69)		8 (31)	18 (69)	
Lymph node metastasis													
Negative	24 (44)	15 (63)	9 (38)	0.032	18 (75)	6 (25)	0.245	15 (63)	9 (38)	0.004	12 (50)	12 (50)	0.777
Positive	30 (56)	10 (33)	20 (67)		18 (60)	12 (40)		7 (23)	23 (77)		8 (27)	22 (73)	
Stage													
I-IIa	22 (41)	14 (64)	8 (36)	0.034	16 (73)	6 (27)	0.433	15 (68)	7 (32)	0.001	10 (45)	12 (55)	0.288
>IIb	32 (59)	11 (34)	21 (66)		20 (63)	12 (37)		7 (22)	25 (78)		10 (31)	22 (69)	

^aThe nuclear expression of proteins in cholangiocarcinoma cells was evaluated; -, negative or <5% of carcinoma cells were positive; +, >5% of carcinoma cells were positive. CDKs, cyclin-dependent kinases. P-CDK1, phosphorylated cyclin-dependent kinase 1.

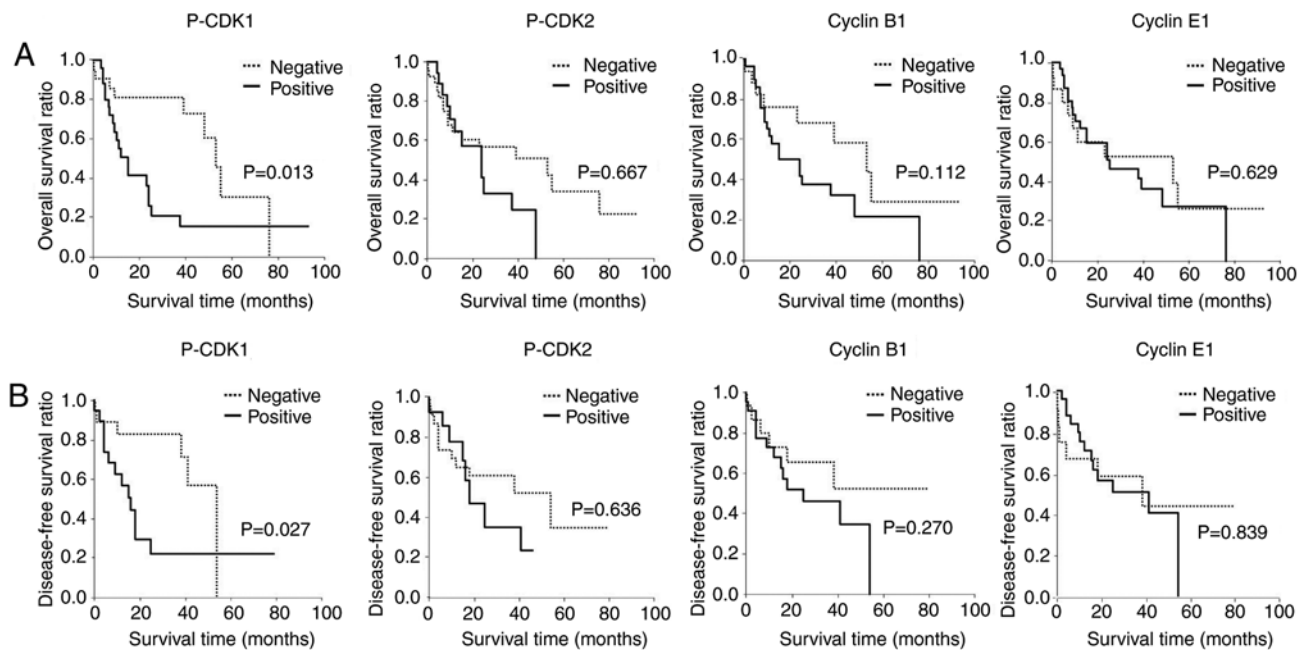


Figure 2. Survival curve of cholangiocarcinoma cases in relation to the immunohistochemical expression of CDKs and cyclins. Patients with positive nuclear expression of p-CDK1 exhibited a significantly worse prognosis in (A) overall survival and (B) disease-free survival. The nuclear expression of p-CDK2, cyclin B1, and cyclin E1 was not associated with patient survival. Survival curves were compared by the log-rank test. CDKs, cyclin-dependent kinases; p-CDK1, phosphorylated cyclin-dependent kinase 1.

Effects of CDK inhibition on cholangiocarcinoma in a murine xenograft model. The subcutaneous implantation of CCKS-1 was used in the analysis of the murine xenograft model. An intraperitoneal injection of roscovitine inhibited tumor growth in this model, and a significant difference was noted in the tumor growth rate between the untreated and treated groups between 10 and 13 days after roscovitine treatment (Fig. 6A and B). Tumors in the untreated group grew up to a volume of 847 mm³ (6.7-fold growth against the initial size), whereas tumors in the treated group had a maximum volume of 600 mm³ (3.0-fold growth against the initial size).

Similar to the results of the *in vitro* experiments, the apoptotic response of cholangiocarcinoma cells was significantly induced by the treatment (Fig. 6C and D). The Ki-67-labeling index of cholangiocarcinoma cells was significantly reduced by the treatment (Fig. 6C and E). Thus, roscovitine was an effective agent to treat cholangiocarcinoma *in vivo*.

Discussion

The present study revealed the significant involvement of the CDK pathway and CDK1 in cholangiocarcinoma. CDK1 and related molecules play key roles in the cell cycle, and the upregulated expression of CDK1 occurs in a number of human malignant tumors (9-12). CDK1 forms a complex with cyclin B1, and the CDK1-cyclin B1 complex (M-phase promoting factor) is indispensable for cell cycle transition into the G2/M phase (27,28). The CDK1 protein is activated by phosphorylation at the Thr161 amino acid activation site and by dephosphorylation at the Thr14/Tyr15 amino acid inhibition sites. CDK1 and cyclin B1 are located in both the nuclei and cytoplasm of cells, and the activation of CDK1-cyclin B1 triggers its rapid accumulation in nuclei (28,29).

The immunohistochemical analysis revealed that the nuclear expression of p-CDK1 (Thr161) and cyclin B1, rather than their cytoplasmic expression, was correlated with clinicopathological factors and/or patient prognosis. These results indicated that the activation of CDK1-cyclin B1 contributes to the aggressive behavior of cholangiocarcinoma. Since the immunohistochemical expression of p-CDK1 and cyclin B1 was absent or weak in normal biliary epithelial cells and hepatocytes, the therapeutic inhibition of the CDK pathway involving CDK1 may have less hepatotoxicity for the normal liver physiology.

Positive correlations have been suggested between CDK phosphorylation and their downstream targets, including cyclins. However, the immunohistochemical analysis revealed no correlations between p-CDK1/cyclin B1 and p-CDK2/cyclin E1 positivity. After the mitotic phase, p-CDK1/cyclin B1 are inactivated by dephosphorylation and the destruction of cyclin B1 (28). The activation of CDK2 requires the assembly of cyclin E and phosphorylation at the Thr160 amino acid activation site by CDK activation enzymes. p-CDK2/cyclin E1 are inactivated by dephosphorylation at the Thr14/Tyr15 amino acid inhibition site by cdc25A, and the destruction of cyclin E occurs via ubiquitination and proteasome processing during the S phase (30,31). Although the exact reason for this currently remains unclear, the complex interactions between CDKs, cyclins, and other related molecules may account for the lack of correlations between p-CDK1/cyclin B1 and p-CDK2/cyclin E1 positivity.

CDK inhibitors exert antitumor effects in various types of cancers, and CDK1 is a target molecule (13-15,32-34). Roscovitine is a broad-range purine inhibitor that inhibits CDK1, CDK2, CDK5, and CDK 7 through direct competition at the ATP-binding site. Roscovitine inhibits cell proliferation

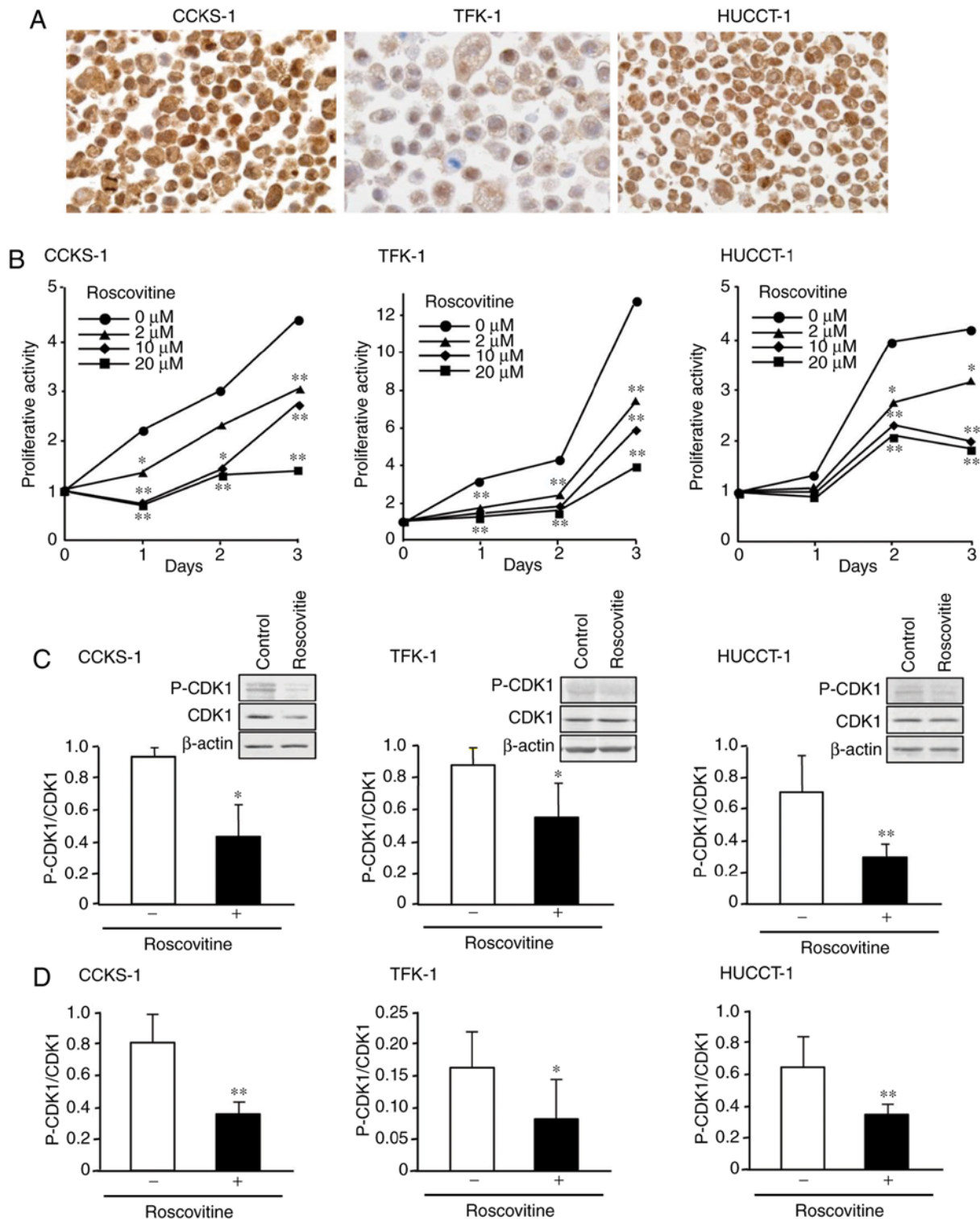


Figure 3. Effects of roscovitine on the cell proliferative activity of cholangiocarcinoma *in vitro*. (A) Immunohistochemical expression of p-CDK1 in the human cholangiocarcinoma cell lines, CCKS-1, TFK-1, and HUCCT-1. (B) The inhibition of the cell proliferative activity of these cell lines following the treatment with roscovitine that was assessed using the WST-1 assay. (C) A western blot analysis indicating the reduced expression of p-CDK1 following roscovitine treatment. The ratio of p-CDK1/CDK1 was determined for each sample. Representative western blots are presented in the upper right corner of each figure, in which the image in each square was captured from the same blotting membrane. (D) An ELISA analysis revealing that roscovitine treatment reduced the ratio of p-CDK1/CDK1 in the cells. Data were expressed as the mean \pm SD of (B) eight, (C) five, and (D) six sets. The significance of differences was assessed using the Student's t-test. * $P < 0.05$, ** $P < 0.01$. Original magnification, $\times 1,000$ (A). p-CDK1, phosphorylated cyclin-dependent kinase 1.

in various cancers, and the anti-proliferative effect is mediated by cell cycle arrest, which is accompanied by the induction of apoptosis (35-38). Regarding the application of roscovitine to biliary diseases, it was revealed to inhibit cholangiocyte growth

(liver cystogenesis) in a mouse model of autosomal dominant polycystic kidney disease (39). Consistent with previously reported findings, it was revealed that roscovitine inhibited the cell proliferation of cholangiocarcinoma, decreased the

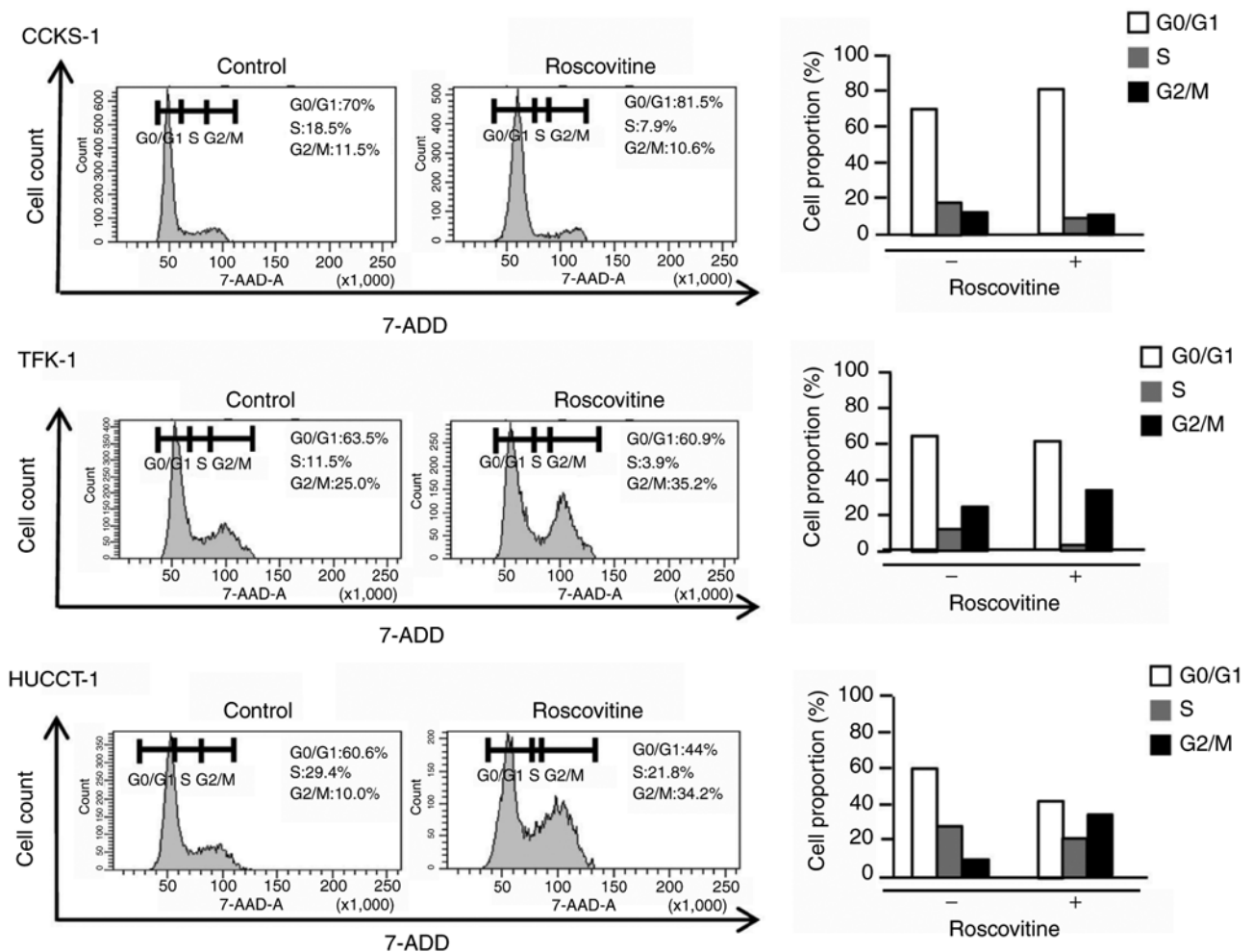


Figure 4. Effects of roscovitine on the cell cycle progression of cholangiocarcinoma *in vitro*. A flow cytometric analysis using the human cholangiocarcinoma cell lines, CCKS-1, TFK-1, and HUCCT-1. The treatment with roscovitine resulted in cell cycle arrest at the G1 or G2-M phase. Data were expressed as the percentage of total cells. Representative images of three independent experiments are presented.

expression of p-CDK1, arrested the cell cycle at the G1 or G2/M phase, and induced apoptosis. Furthermore, roscovitine inhibited the invasion of cholangiocarcinoma cells *in vitro*. The activation of CDK5 was revealed to be involved in the invasion of PDAC, and the inhibition of cholangiocarcinoma cell invasion may be associated with the inhibitory effects of roscovitine on CDK5 activation (40).

In vivo studies further confirmed that roscovitine significantly inhibited cell proliferative activity, and induced apoptosis in a murine xenograft model of cholangiocarcinoma. These results were consistent with the data of previous studies that demonstrated the antitumor effects of roscovitine *in vivo* (25,37,41). It has been reported that roscovitine induced apoptosis in a xenograft model of Ewing's sarcoma family tumor by a caspase-dependent mechanism (25). Another study revealed that roscovitine enhanced the antitumor effects of doxorubicin by inducing G2/M arrest rather than apoptosis accompanied by the increase of p27 expression in a breast cancer xenograft model (41). Although the detailed mechanism requires study, it is plausible that the xenograft growth inhibition by roscovitine relates to the complex interaction of molecules included in different signaling pathways, and the inhibition of cell cycle progression and the induction of

apoptosis may be important factors attributable to the anti-tumor effects of roscovitine *in vivo*. The inhibition of tumor invasion may also contribute to the antitumor effects.

KRAS mutant tumor cells have elevated CDK1 activity, and CDK1 may be a synthetic lethal target for KRAS mutant tumors including PDAC (16). Activating KRAS mutations are frequently observed in cholangiocarcinoma predominantly of the perihilar and distal types and are reported to be present in up to 40% of cases (17-19). CCKS-1 and HUCCT-1 were revealed to harbor a KRAS mutation (G12D), whereas TFK-1 had no mutation at codon 12 of KRAS (42,43). The stronger nuclear expression of p-CDK1 in CCKS-1 and HUCCT-1 than in TFK-1 may reflect the presence of KRAS mutations in cells, and suggests that CDK1 is a preferable therapeutic target for cholangiocarcinoma with KRAS mutations.

In the present study, the nuclear expression of p-CDK1 was also observed in TFK-1. Although p-CDK1 expression in TFK-1 was weak on immunostaining, the presence of p-CDK1 indicates its potential as a therapeutic target. The multi-CDK inhibitor roscovitine inhibited cell proliferative activity and arrested the cell cycle in TFK-1 as well as in CCKS-1 and HUCCT-1; however, the extent of the involvement of CDK1 inhibition in these inhibitory effects was

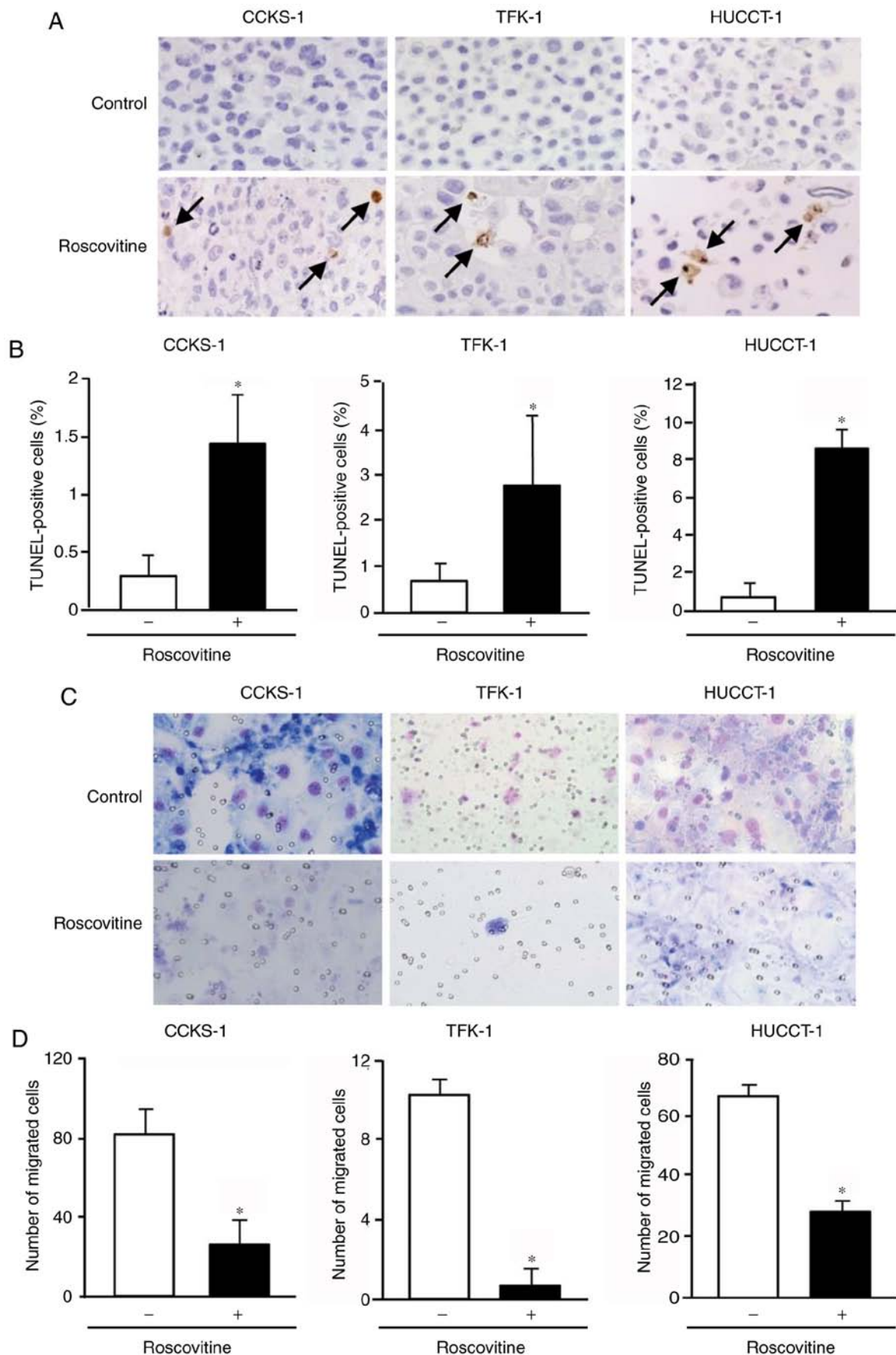


Figure 5. Effects of roscovitrine on apoptosis and cell invasion of cholangiocarcinoma *in vitro*. (A) Representative microphotographs of the TUNEL assay for the cholangiocarcinoma cell lines, CCKS-1, TFK-1, and HUCCT-1. Arrows indicate TUNEL-positive cells. (B) Induction of apoptosis in cells following roscovitrine treatment. (C) Representative microphotographs of the invasion assay. (D) Inhibition of cell invasion following roscovitrine treatment. Data were expressed as the mean \pm SD of five sets (B and D). The significance of differences was assessed using the Student's t-test. * $P < 0.05$. Original magnification, $\times 1,000$ (A and C). TUNEL, terminal deoxynucleotide transferase-mediated dUTP nick end-labeling.

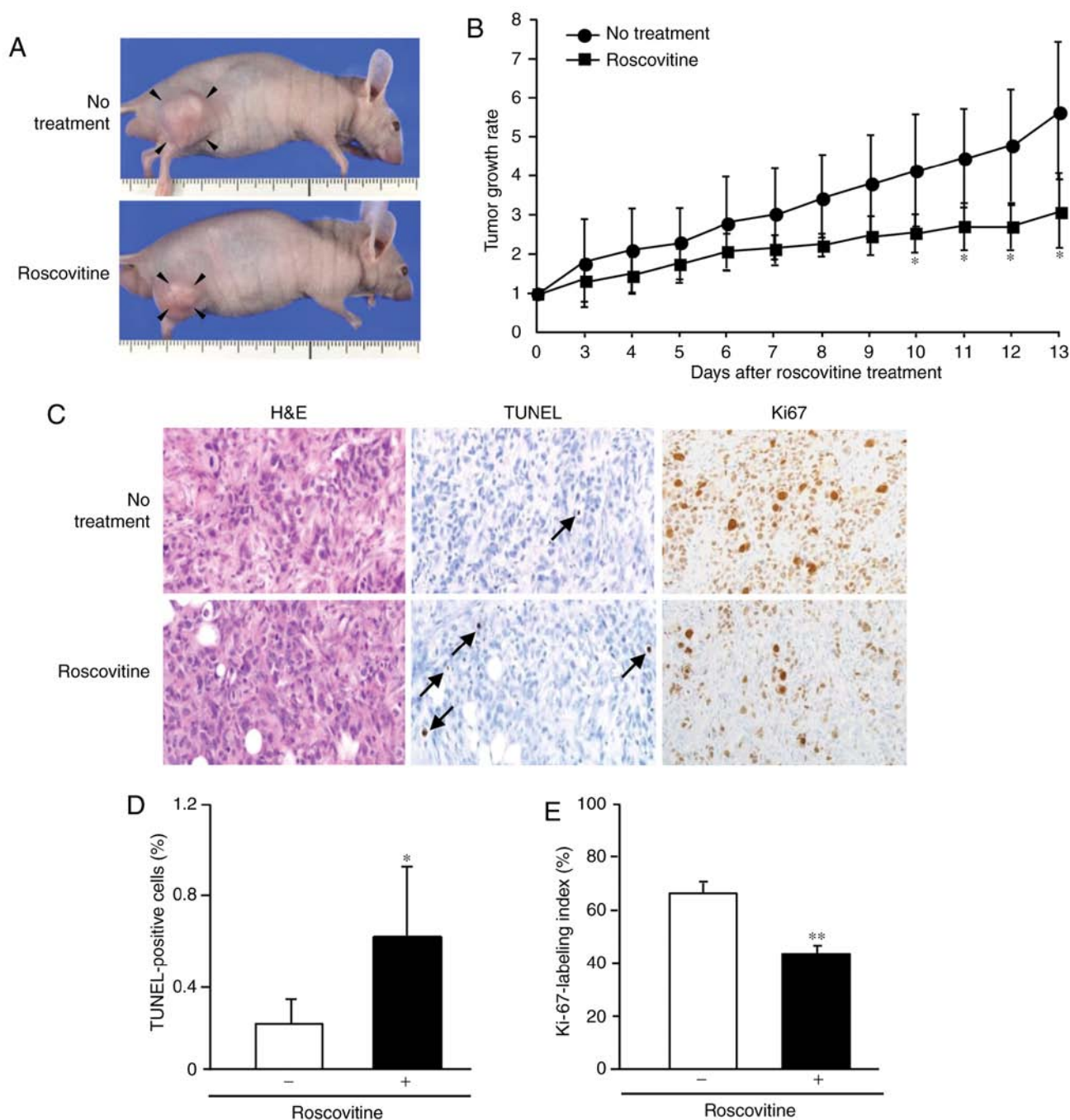


Figure 6. Effects of roscovitine on cholangiocarcinoma in a murine xenograft model. (A) Representative images of a murine xenograft model established by the subcutaneous implantation of the human cholangiocarcinoma cell line CCKS-1. Arrowheads indicate tumor nodules. (B) Time course of tumor growth in non-treated and roscovitine-treated mice. (C) Representative microphotographs of tumor tissues obtained from non-treated and roscovitine-treated mice. Arrows indicate TUNEL-positive cells. (D) Induction of apoptosis in cells following the roscovitine treatment. (E) Inhibition of cell proliferation following roscovitine treatment as assessed by the Ki-67-labeling index. Data were expressed as the mean \pm SD of five sets (B, D, and E). The significance of differences was assessed using the Student's t-test. * $P < 0.05$, ** $P < 0.01$. Original magnifications, $\times 400$ (C). TUNEL, terminal deoxynucleotide transferase-mediated dUTP nick end-labeling.

unclear, and the effects of roscovitine on other CDKs may be involved. Therefore, studies using specific inhibitors of CDK1 are required to further clarify the biological significance of CDK1 in cholangiocarcinoma. Furthermore, the relationship between the KRAS mutation status and the extent of p-CDK1 expression needs to be analyzed in patients with cholangiocarcinoma.

In summary, the present study demonstrated that the pharmacological inhibition of CDK pathways using roscovitine

reduced cell proliferation and the invasion of cholangiocarcinoma. These inhibitory effects were accompanied by cell cycle arrest at the G1 or G2/M phase and the induction of apoptosis. The immunohistochemical expression of p-CDK1 and cyclin B1 predicted the aggressive behavior of cholangiocarcinoma, and p-CDK1 may be a useful prognostic marker of cholangiocarcinoma. These results indicated that the CDK pathway involving CDK1 has potential as a therapeutic target for cholangiocarcinoma.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MY performed the experiments and wrote the manuscript with technical support from KT. YS and KH conceived and designed the study. MS analyzed the data and provided comments. All authors participated in data interpretation and critically reviewed and approved the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Experiments using human materials were performed with the approval of the Ethics Committee of Kanazawa University Graduate School of Medicine (Permit no. 1985-3). Protocols for animal studies were approved by the Committee of the Institute for Experimental Animals, Kanazawa University Advanced Science Research Center (Permit no. AP-173905).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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