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CDKN3 acts as a tumor promoter enhancing proliferation of cholangiocarcinoma cells

Orawan Waenphimai[a,†], Ubonrat Thamrongwaranggoon[a,†], Sonexai Kidoikhammouan[b], Supannika Sorin[a], Nattakarn Klinhom-on[a], Wunchana Seubwai[c,d], Charupong Saengboonmee[a,d], Marutpong Detarya[a], Prin Sungwan[b], Sopit Wongkham[a,d], Worachart Lert-itthiporn[a,d,*]

[a] Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002 Thailand
[b] Biomedical Science Program, Graduate School, Khon Kaen University, Khon Kaen 40002 Thailand
[c] Department of Forensic Medicine, Faculty of Medicine, and Cholangiocarcinoma Research Institute, Khon Kaen University, Khon Kaen 40002 Thailand
[d] Center for Translational Medicine, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002 Thailand

*Corresponding author, e-mail: woracle@kku.ac.th
† These authors contributed equally as the co-first author.

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ABSTRACT: Cholangiocarcinoma (CCA) is a biliary tract tumor with high metastasis, often diagnosed at advanced stages when surgical treatment is challenging. Cyclin-dependent kinase inhibitor 3 (CDKN3) is a member of the dual-specificity protein phosphatase family that can dephosphorylate CDK2, thus preventing the activation of CDK2 and suppressing cell proliferation. Increased or decreased CDKN3 expression was reported in different cancer types with a debatable role of tumor suppressor or promoter. The expression and roles of CDKN3 in CCA have never been explored. The aim of this study was to clarify for the first time the oncogenic role of CDKN3 in CCA. GEO database analysis revealed a substantially significant expression of CDKN3 mRNA in CCA tissues, regardless of tumor type, location, and etiology. However, the clinical impacts of CDKN3 expression on clinico-pathological features and the overall survival of CCA patients were not clearly seen. In contrast, *in vitro* study showed that CDKN3 gene silencing with siRNA dramatically decreased proliferation and colony formation of CCA cell lines. Western blot analysis revealed that expression of cell proliferation markers, cyclin D1 and p21, was significantly reduced when CDKN3 was suppressed. The combination treatment of siCDKN3 with gemcitabine significantly enhanced the cytotoxic effect of gemcitabine against CCA and gemcitabine-resistant CCA cells, as shown by the values of combination index and dose reduction index retrieved from the isobologram. The results of this study address the oncogenic functions of CDKN3 in CCA and identify it as a viable target for CCA therapy.

KEYWORDS: cyclin D1, gemcitabine resistance, isobologram, combination index

INTRODUCTION

Cholangiocarcinoma (CCA), a rare bile system malignancy, is rapidly becoming more common throughout the world (reviewed in Brindley et al) [1]. In the Greater Mekong subregion, *Opisthorchis viverrini* infection is a recognized risk factor for CCA, whereas in Western nations, chronic biliary tract inflammation, including cholelithiasis, choledocholithiasis, and primary chronic sclerosing cholangitis, are the risk factors for CCA. Regardless of the cause, CCA is typically detected in its advanced stages, when surgical cures cannot be offered. The need for innovative CCA treatment options is growing because of the disease's rising incidence and few available effective treatments.

Cyclin-dependent kinase inhibitor 3 (CDKN3, also called CDI1 or KAP) is a member of the dual-specificity protein phosphatase family that can specifically dephosphorylate and inactivate CDK2. Thus, CDKN3 functions as a significant inhibitor of cell cycle progression. With debatable evidence, CDKN3 might act as either a tumor suppressor or an oncogenic protein in a number of malignancies. It was described as a tumor suppressor in glioblastoma [2], hepatocellular carcinoma [3], and Bcr-Abl-mediated chronic myelogenous leukemia [4]. Overexpression of CDKN3 sensitized the leukemic cells to imatinib-induced apoptosis and dramatically inhibited tumor growth in the xenografted mouse model. However, significant increases in CDKN3 expression were found in breast cancer [5], esophageal squamous cell carcinoma [6], and colorectal cancer [7], and the role of CDKN3 was considered as a tumor promotor. Overexpression of CDKN3 promoted proliferation, colony formation, migration, and invasion of esophageal squamous cell carcinoma; while CDKN3 gene silencing promoted apoptosis, induced G1 phase cell cycle arrest, and decreased cell migration of breast cancer cells [5]. The molecular mechanism of CDKN3 was suggested to involve p27/AKT, as shown in nasopharyngeal carcinoma [8] and esophageal squamous cell carcinoma [9]. In addition, high levels of CDKN3 expression in hepatocellular carcinoma were linked to larger tumors, more advanced clinical stages, and worse patient out-

comes [10]. Furthermore, the increase of CDKN3 expression was linked to poor prognosis in human lung adenocarcinoma and cervical cancer [11, 12]. These pieces of evidence imply the possibility of CDKN3 as a novel target of cancer treatment.

It remains unclear if CDKN3 functions as a tumor promoter or a tumor suppressor in CCA. Here, we demonstrated that CDKN3 was elevated in tumor tissues of CCA patients and contributed to the malignancy of CCA. Using CCA cell lines, CDKN3 gene silencing decreased cellular proliferation and colony formation and sensitized the cytotoxicity of gemcitabine in gemcitabine-resistant CCA cells. The results of this study demonstrated that CDKN3 as a potential new therapeutic target for the treatment of progressive CCA.

MATERIALS AND METHODS

Cell lines and cell culture

KKU-055, KKU-100, KKU-213A, and KKU-213B cell lines were acquired from the Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan. All cell lines were established from the primary CCA tissues of Thai patients with different histological subtypes. KKU-055 and KKU-100 were from poorly differentiated tubular adenocarcinoma [13], whereas KKU-213A and KKU-213B were from CCA with poorly differentiated- and well differentiated-squamous cell carcinomas [14]. KKU-213A-GemR cells were established by Thamrongwaranggoon et al [15]. All CCA cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA) with 25 mM glucose and 10% fetal bovine serum (FBS, Gibco), at 37 °C and 5% CO_2. The protocol for this study (HE641576) was approved by the Ethics Committee for Human Research of Khon Kaen University.

siCDKN3 treatment

CDKN3 mRNA expression in CCA cells was transiently suppressed using RNA silencing as previously described [12]. A pooled siRNA specifically targeted CDKN3 (10 pmole) (SC-43877, Santa Cruz, Dallas, Texas, CA) and 1 µg/µl Lipofectamine®2000 (Thermo Fisher Scientific, Waltham, MA, USA) were applied. Cells treated with scrambled siRNA (Qiagen, Hilden, Germany) in the same manner were used as a negative control.

Cell viability measurements

In brief, 1.5×10^3 cells/well of siCDKN3 or scrambled siRNA-treated cells were cultured in a 96-well plate for 24, 48, and 72 h. Cell viability was determined using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay (Invitrogen, Waltham, MA, USA) as previously described [15].

Clonogenic assay

CCA cells were treated with siCDKN3 or scrambled siRNA for 24 h, trypsinized, and seeded into a 24-well plate at 100 cells/well. The colony formation was assessed 7 days post-treatment as previously described [16].

Combination of siCDKN3 and gemcitabine in CCA treatment

KKU-213A and KKU-213A-GemR cells (2×10^3 cells/well) were cultured in a 96 well plate for 16 h, then treated with siCDKN3 in the FBS-free medium for 6 h, and cultured further in the complete medium for 18 h. For combination treatment, cells were treated with siCDKN3 (0.25 µM) in combination with IC_{25}, IC_{45}, and IC_{65} doses of gemcitabine of either KKU-213 or KKU213A-GemR for 72 h as indicated in Table 1. Cell viability was determined using an MTT assay. The combination index (CI) theorem of Chou-Talalay [17] was used to determine the additive effect ($CI = 1$), synergism ($CI < 1$), and antagonism ($CI > 1$) in the combination treatment. Cells treated with siCDKN3 and gemcitabine were analyzed in comparison to the single-agent treated cells. All data were analyzed using COMPUSYN Software (ComboSyn, Paramus, NJ, USA).

SDS-PAGE and Western blotting

Protein content of the cell lysates in RIPA lysis buffer (50 mM This-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1% Na-deoxycholate, and 0.1% SDS) was determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). Western blots and semi-quantitative analyses of the target proteins were performed according to the methods described in Thamrongwaranggoon et al [15]. Cell lysate (30 µg of protein) was loaded on a 12% SDS-PAGE and transferred to a PVDF membrane (Merck Millipore, Burlington, MA, USA). The membrane was incubated with 1:1000 dilution of primary antibodies, except otherwise specified, at room temperature for 1 h and at 4 °C overnight: CDKN3 (#MA5-25690) and phospho-CDK2 (#PA5-77906) from Thermo Fisher Scientific; CDK2 (#10122-1-AP) and 1:2000 PCNA (#24036-1-AP) from Proteintech, Rosemont, IL, USA; cyclin D1 (#2978S) and p21 (#2947S) from Cell Signaling Technology, Danvers, MA, USA; and 1:5000 mouse anti-GAPDH (#MAB374) from Merck Millipore; followed by incubation with HRP-coupled secondary antibody, goat anti-mouse IgG secondary antibody, HRP (dilution 1:5000, #62-6520) from Thermo Fisher Scientific, or anti-rabbit IgG, HRP-linked antibody (dilution 1:2000, #7074S) from Cell Signaling Technology, at room temperature for 1 h. The signals were assessed using Immobilon® Forte Western HRP Substrate (Merck Millipore) as substrate. The chemiluminescent signal was detected using the Amersham ImageQuant LAS 600-image analyzer (GE

Healthcare, Chicago, IL, USA) and quantified using Image J analysis software.

Gene expression profiling

The expression levels of CDKN3 mRNA of CCA tissues were retrieved from the Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/). The GSE76297 dataset was from patients with intrahepatic CCA, composing of 91 CCA tissues and 92 adjacent non-tumor tissues [18], and the GSE26566 dataset was from CCA cases with extrahepatic (hilar and peripheral types) CCA of 104 CCA tissues and 65 non-tumor tissues [19]. Dataset GSE89749 was retrieved from 48 CCA patients [20]. Pearson's correlation coefficient was used to determine the relationship between the expression of CDKN3 and the cell proliferation markers: cyclin D1 (CCND1), p21, and proliferating cell nuclear antigen (PCNA) from the GSE76297 dataset. The survival time and clinical features of CCA cases were obtained from the GSE89749 dataset. Univariate and multivariate analyses were done using Cox regression analysis. Survival analysis was performed using the Kaplan-Meier method and log-rank test.

Statistical analysis

All statistical analyses were performed using SPSS 26 software (SPSS, Chicago, IL, USA). All experiments were performed in three independent experiments. Data were presented as mean ± standard deviation (SD), and the differences between groups were compared using the Student's *t*-test. The data with a p-value < 0.05 was considered significant.

RESULTS

CDKN3 was upregulated in CCA tissues

The differential expression of CDKN3 in human CCA tissues was first analyzed using the datasets GSE76297, GSE26566 and GSE89749 from GEO. As shown in Fig. 1a, CCA tissues had considerably higher CDKN3 expression than their non-tumor counterparts, regardless of the sources of the dataset. There was no statistically significant difference between patients with low and high CDKN3 expression in CCA tissues in terms of overall survival (Fig. 1b). According to the univariate analysis on the GSE89749 dataset, the clinical characteristics (gender, age, histological type, and tumor stage) of CCA patients were not linked with the expression of CDKN3 (Table S1). As CDKN3 inactivates CDK2 in cell cycle, whether upregulation of CDKN3 is related to the expression of CDK2 and other key players in the cell cycle was analyzed, e.g., cyclin D1 in G1 phase, CDK2 in G1/S, p21 (CDK2 and CDK1 inhibitors), and proliferative markers, PCNA. It was found that CDKN3 was positively correlated with that of cyclin D1 (CCND1, Fig. 1c) and PCNA (Fig. 1e), while it was reversely correlated with the expression level of p21 (CDK1A, Fig. 1d). These results implied the mediation of CDKN3 in CCA proliferation. Similar to CDKN3, aldehyde dehydrogenase 1 family member A3 (ALDH1A3) was also upregulated in CCA tissues and high in KKU-213A-GemR cell line [15]. Whether CDKN3 associates with ALDH1A3 in supporting gemcitabine resistance in CCA was then explored. Using GSE76297 dataset, no correlation between the expression of CDKN3 and ALDH1A3 was observed, suggesting that they might mediate gemcitabine resistance through independent mechanisms (Fig. S1).

CDKN3 gene silencing suppressed proliferation and colony formation of CCA cells

Endogenous expression of CDKN3 in four human CCA cell lines established from primary tumors with different histological subtypes was determined using Western blotting (Fig. 2a). CDKN3 expression was variable among four CCA cell lines; strongly expressed in KKU-213A and KKU-213B, moderately in KKU-100, and weakly in KKU-055. Then, KKU-213A and KKU-213B cell lines with strong and moderate CDKN3 expression were selected for the subsequent CDKN3 gene silencing studies.

The expression levels of CDKN3 in KKU-213A and KKU-213B were transiently silenced down to $< 25\%$ of the scramble control cells within 24 h after siCDKN3 treatment (Fig. 2b,c). CDKN3 gene suppression did not affect cell morphology (Fig. 3a), but significantly suppressed cell proliferation of both cell lines at 48 and 72 h ($p < 0.001$; Fig. 3b). siCDKN3-treatment also suppressed the colony formation in CCA cell lines to significantly lower than that of the scramble control cells ($p < 0.01$; Fig. 3c). The results indicated the promoting effect of CDKN3 on CCA cell growth.

To further validate the inhibitory effect of siCDKN3 treatment on cell proliferation, cell cycle phases of KKU-213A and KKU-213B cells 48 h after treatment with siCDKN3 or scramble RNA were analyzed using flow cytometry. As shown in Fig. S2, suppression of siCDKN3 treatment did not affect the cell cycle phases.

CDKN3 modulated the expression of cell proliferation-related genes

To elucidate further the molecular mechanisms related to the mode of action of CDKN3 to promote CCA cell proliferation, expression levels of several target proteins related to CDKN3 action and cell proliferation in siCDKN3 treated and scramble control cells were assessed using Western blotting. To identify CDKN3-associated signaling pathways, expression levels of pCDK2 and CDK2 were assessed for CDKN3 activities, whereas cyclin D1, p21, and PCNA expressions were assessed for cell proliferation. The Western blots and semi-quantitative data shown in Fig. 4a,b demonstrated that siCDKN3 treatment for 48 h significantly decreased the expression of CDKN3, cyclin D1, and p21; while it increased the expression of pCDK2. The

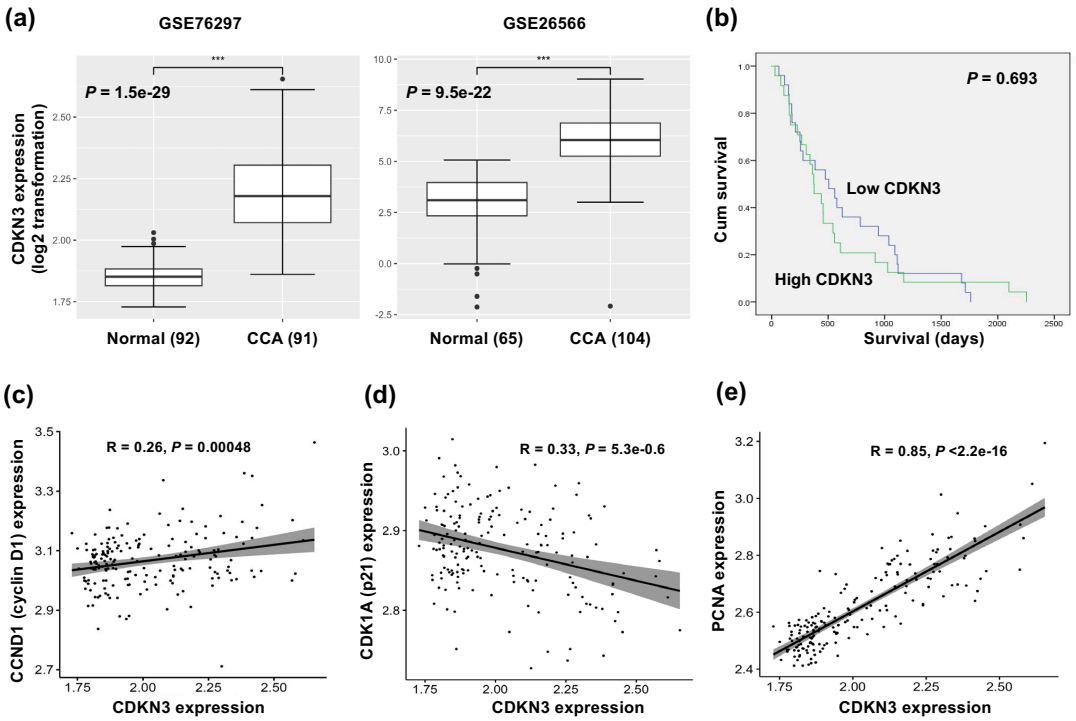

Fig. 1 CDKN3 expression in CCA tissues and its relation to cell proliferation makers. (a), CDKN3 expression level in intrahepatic and extrahepatic CCA tissues; (b), survival analysis of CCA patients with high and low CDKN3 expression in CCA tissues based on the median as a cut-off; (c), (d), and (e), correlation between CDKN3 expression level and the cell proliferation markers cyclin D1, p21, and PCNA in CCA tissues, respectively. *** $p < 0.001$.

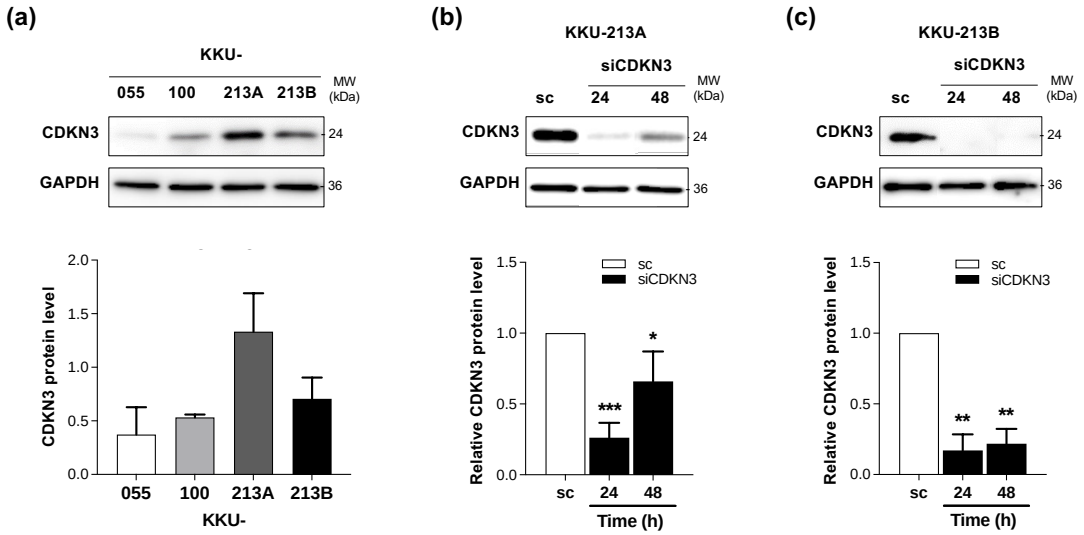

Fig. 2 Western blot analysis of CDKN3 expression in CCA cell lines with bar graphs displaying the ratio of CDKN3 to GAPDH. (a), Endogenous expression in four CCA cell lines; (b), expression in KKU-213A cells; and (c), expression in KKU-213B cells. Data were mean ± SD from three independent experiments by giving those of the control as 1. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

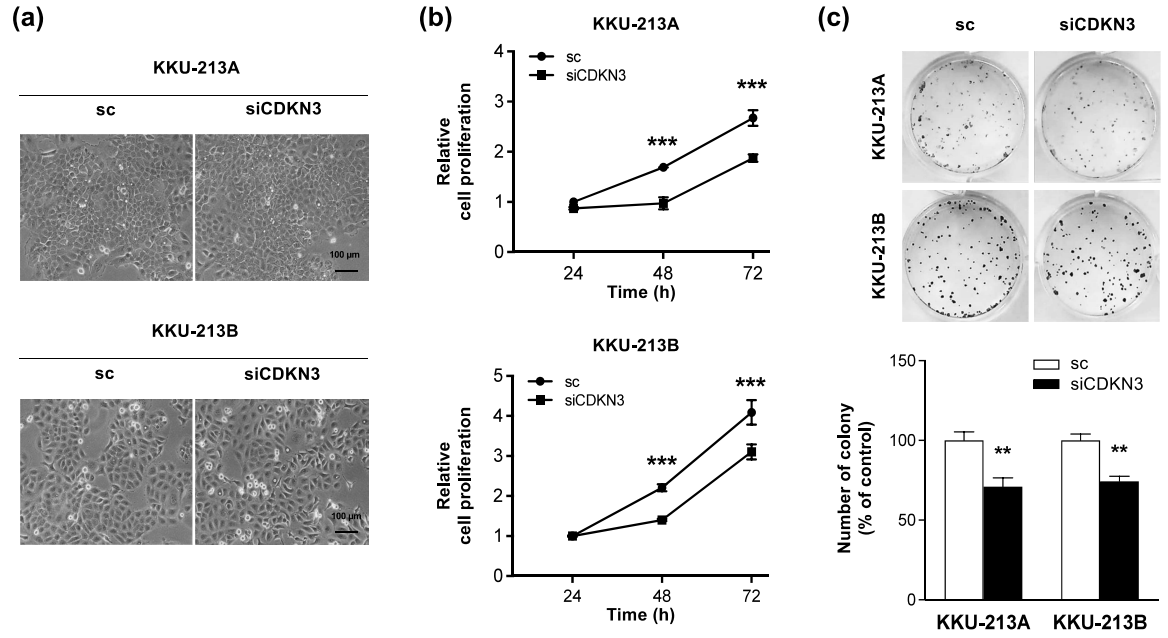

Fig. 3 Effects of siCDKN3 treatment on cell proliferation and colony formation of CCA cell lines KKU-213A and KKU-213B. (a), Cell morphology 48 h after siCDKN3 treatment (phase-contrast, scale bar = 100 microns); (b), cell proliferation rates; and (c), colony formation. Data were mean ± SD of three independent experiments. ** $p < 0.01$ and *** $p < 0.001$.

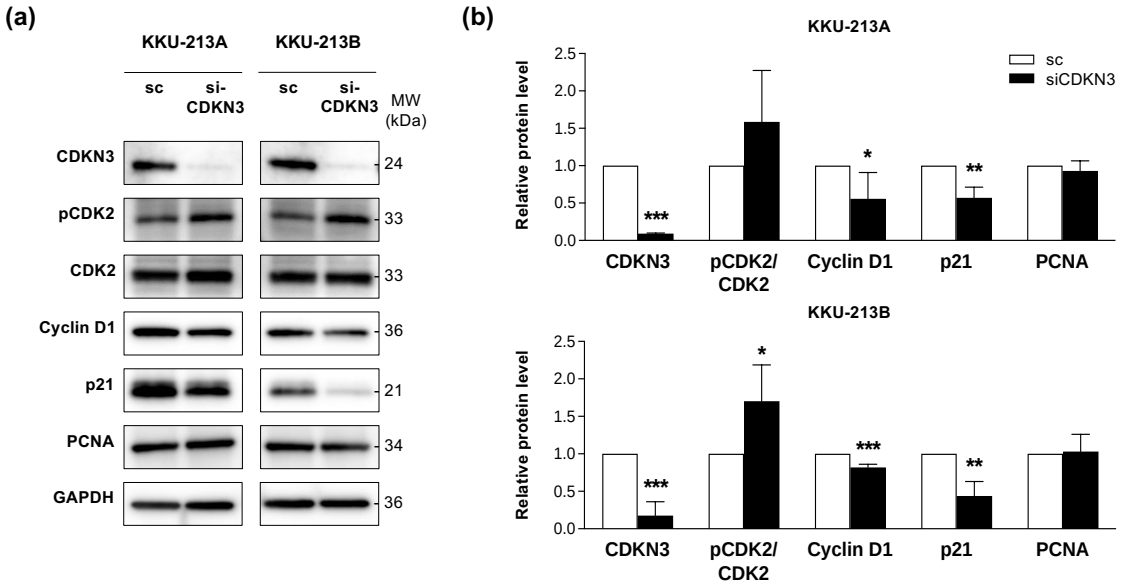

Fig. 4 Effects of CDKN3 on expression of cell proliferation-related genes of CCA cell lines KKU-213A and KKU-213B treated with siCDKN3 or scramble control for 48 h. (a), Western blotting; and (b), semiquantitative analysis. Data were mean ± SD from three independent experiments, giving value of the control as 1. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

treatment, however, did not affect the expression levels of CDK2 and PCNA. The results, hence, implied that CDKN3 influenced the proliferation rate of CCA cells at least in part via those cell cycle regulating proteins.

Suppression of CDKN3 expression sensitized CCA cells to the cytotoxicity of gemcitabine

Drug combination treatment is currently an approach to increase the efficiency of chemotherapy and to

Table 1 Effects of siCDKN3 on the combination index and dose reduction index of gemcitabine.

Cell line	siCDKN3:GEM (IC)	siCDKN3 (μM)	GEM (μM)	Fa	CI	DRI GEM
KKU-213A	siCDKN3:25	0.25	0.2	0.50 ± 0.03	0.51	2.0
	siCDKN3:45	0.25	0.3	0.60 ± 0.05	0.67	1.5
	siCDKN3:65	0.25	0.5	0.74 ± 0.02	0.62	1.6
KKU-213A-GemR	siCDKN3:25	0.25	2.0	0.52 ± 0.04	0.45	2.2
	siCDKN3:45	0.25	3.7	0.59 ± 0.02	0.73	1.3
	siCDKN3:65	0.25	5.3	0.67 ± 0.01	0.86	1.1

GEM, gemcitabine; IC, inhibitory concentration; Fa, fraction affected; CI, combination index; DRI, dose reduction index.

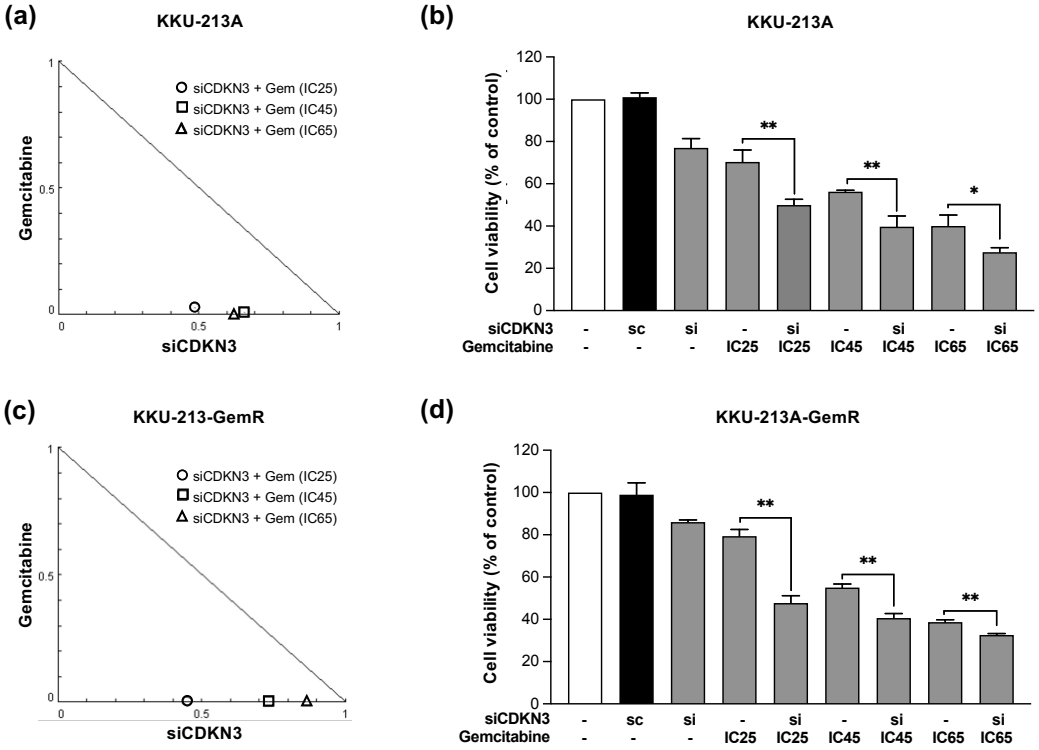

Fig. 5 Synergism of CDKN3 suppression with gemcitabine cytotoxicity against CCA cells. (a) and (c), Isobolograms demonstrating the synergistic effects of CDKN3 silencing with all doses of gemcitabine tested with the CI value < 1; (b) and (d), combination treatment of siCDKN3 enhancing the sensitivity of both KKU-213A and KKU-213A-GemR cells to the cytotoxicity of gemcitabine. Data were mean ± SD from three independent experiments with triplicate assays each. * $p < 0.05$, ** $p < 0.01$.

reduce drug resistance. Thus, we explored whether siCDKN3 treatment could sensitize CCA cells to the cytotoxicity of gemcitabine. For this purpose, parental KKU-213A cells and gemcitabine-resistant KKU-213A-GemR cells, having IC_{50} value of gemcitabine approximately 10–12 folds higher than that of the parental cells, were cultured in the IC_{25}, IC_{45}, or IC_{65} dose of gemcitabine with the presence of scramble RNA or siCDKN3 for 72 h. The viable cells after treatment were determined using MTT assay. The combination index (CI) and the dose reduction index (DRI) values were analyzed (Table 1). The constructed isobologram of siCDKN3-treated KKU-213A and KKU-213A-GemR cells with all concentrations of gemcitabine tested indicated that the CI values of all combinations were < 1 (Fig. 5a,c). The growth inhibitory effect of cells treated with both siCDKN3 and gemcitabine was more prominent than those treated with gemcitabine alone (Fig. 5b,d). The combination treatment reduced the IC_{50} values for gemcitabine of the KKU-213A-GemR and the parental KKU-213A cells from 4.37 and 0.35 μM to 2.05 and 0.2 μM, respectively. As shown

in Table 1, siCDKN3 treatment reduced the DRI values 1-2-folds of gemcitabine required to obtain the similar cytotoxic effect to the cells treated with gemcitabine alone. The synergistic effects were consistently observed in both KKU-213A and KKU-213A-GemR cells. These results signified the sensitizing effect of siCDKN3 on CCA cells to the cytotoxicity of gemcitabine.

DISCUSSION

Although CDKN3 is a crucial negative regulator of cell cycle progression, it can operate as either a tumor suppressor [2–4] or a tumor promoter [5–7] in various malignancies. This study identified for the first time CDKN3 as a tumor promoter in CCA. CDKN3 was overexpressed in patient-CCA tissues compared with the surrounding normal tissues. When CCA cell lines were treated with siCDKN3, cell proliferation and colony formation were significantly reduced. Also, suppression of CDKN3 expression could enhance the sensitivity of parental and gemcitabine-resistant CCA cells to the cytotoxic action of gemcitabine. These results suggested that CDKN3 could be a valuable prospective target for the therapy of CCA.

High expression of CDKN3 has been frequently reported in various cancers [6, 7]. While CDKN3 upregulation is linked to tumor suppression in glioblastoma [2] and breast cancer [5], it is linked to an oncogenic activity that promotes cell proliferation in colorectal cancer [7] and esophageal squamous cell carcinoma [6]. This discrepancy might be caused by the multiple regulatory roles of CDKN3 in the cell cycle of various tumor types.

CDKN3 is a negative regulator of CDK1 and CDK2 by dephosphorylation activity [21, 22]. CDKN3 binds to the substrate and the ATP binding activities of the CDKs and consequently reduces CDKs activities [21]. Dephosphorylation of CDK2 induces problems in DNA replication and genome integrity [23], and thus inhibits cell proliferation. According to this perception, the upregulated CDKN3 acts as a potential tumor suppressor. Alternatively, increase of CDKN3 leads to a decrease of CDK1-cyclin B activity, which is required for mitotic exit [2, 24]. Thus, the decrease in CDK activity is important for the completion of cell division. Knockdown of CDKN3 leads to a mitotic failure [2]. According to these findings, CDKN3 overexpression functions as a strong tumor promoter in cancer cells. CDKN3 contributes to the higher mitotic activity for safe passage through the cell cycle. The mechanism underlying the oncogenic role of CDKN3 in CCA cell proliferation as seen in the present study remains unclear and requires more in-depth explanation.

Numerous studies have been conducted on the role of CDKN3 in cell growth regulation. In the current work, cell proliferation and colony formation of CCA cells were considerably reduced when CDKN3 expression was suppressed. However, no correlation was observed between CDKN3 expression and clinicopathological characteristics, nor with the overall survival of CCA patients. Suppression of CDKN3 expression dramatically reduced the cancer cell growth without affecting the clinical characteristics or overall survival of the patients was reported in esophageal cancer [6]. On the contrary, the linkage between elevated CDKN3 expression and short survival (within 2 years) was reported in cervical cancer patients [12].

Several studies indicated a direct impact of CDKN3 on the cell cycle of cancer cells. Although siCDKN3 treatment had no clear effects on the cell cycle of CCA cells, the results of proliferation and colony formation assays revealed that siCDKN3 treatment caused a significant reduction of CCA cell growth. According to several previous works, siCDKN3 treatment caused the G1 arrest of the cells, while CDKN3 overexpression had opposite effects [6, 7, 25]. In line with previous research [6, 9], CDKN3 silencing of CCA cells lowered cyclin D1 expression levels. In agreement with these findings, CCA tissues of patients showed that CDKN3 expression was positively correlated with cyclin D1 and negatively correlated with p21. In accordance with the observed effects of siCDKN3 on cell proliferation, it is essential to note the decrease in cyclin D1 levels as observed in the Western blot analysis. Cyclin D1 is a crucial regulator of the cell cycle, specifically in the transition from G1 to S phase, and its reduction aligns with the inhibition of cell proliferation following siCDKN3 treatment. Decrease in cyclin D1 level further supports the hypothesis that CDKN3 plays a significant role in promoting cell proliferation in CCA. These results suggest that targeting CDKN3 could disrupt the critical process of cell cycle, thereby inhibiting tumor growth and enhancing the efficacy of chemotherapeutic agents like gemcitabine. *In vitro* and *in vivo* experiments with pancreatic cancer cells demonstrated that CDKN3 could form a complex with MdM2-P53, and thus inhibited p21-expression [26]. On the other hand, p21 could promote oncogenesis in a p53-independent manner [27, 28]. In the context of DNA damage, p21 inhibits apoptosis and promotes cell survival, leading to the accumulation of genetic mutations and tumorigenesis [29]. Additionally, p21 enhances the process of cell migration, invasion, and epithelial-mesenchymal transition, which are critical for cancer metastasis by interacting with signaling molecules and cytoskeletal components [30]. It was suggested that p21 might encourage cell proliferation when its concentration reached a particular threshold. These results might justify the reduction of p21 expression seen in siCDKN3-treated CCA cells in the current study. Other nucleosome-binding proteins, such as HMGN3, demonstrate similar effects in CCA, promoting tumor aggressiveness and invasion [31]. This highlights the complexity of cell cycle regulation and emphasizes the importance of cellular context in

interpreting results, warranting further investigation into the p53 status of our cell lines and alternative regulatory mechanisms [26, 32]. In addition, as CDKN3 expression varies in different types of malignancies, the function and regulation of CDKN3 in the cell cycle may also differ based on the cancer type. To understand the roles of CDKN3 in cell cycle regulation, a deeper investigation of the activity and participation of CDKN3 in each phase of cell cycle is needed.

Currently, gemcitabine and gemcitabine plus cisplatin are the first-line treatment recommended for advanced CCA [1, 33, 34]. Gemcitabine blocks the progression of the G1/S phase and induces cell apoptosis [35, 36]. Information on molecular mechanism of gemcitabine resistance in CCA is rather limited. A mechanism involved downregulation of equilibrative nucleoside transporters, affecting gemcitabine uptake was reported [37]. Several drug combination treatments were proposed to improve the effectiveness of gemcitabine and lower drug resistance since acquired resistance causing a poor response to gemcitabine was frequently seen in CCA patients [15, 38]. A growing body of evidence suggests that CDKN3 expression in cancer cells and patients is either favorably or negatively associated with chemo-resistance, e.g., bladder cancer [39], colorectal cancer [40] and hepatocellular carcinoma [10], etc.

Recently, CDKN3 has been included in the eleven up-regulated hub genes considered as a novel candidate of targets for drug development for CCA therapy [41]. In this study, siCDKN3 treatment exhibited a synergistic effect on gemcitabine toxicity against parental KKU-213A and gemcitabine-resistant KKU-213A-GemR cells. The findings from the aforementioned studies and the present study suggested that CDKN3 can be a potential target for CCA treatment. Clarification of the role of CDKN3 in CCA is required to design an appropriate strategy to target CDKN3. Additionally, more research is required to find or create a small molecule and an effective delivery strategy for introducing CDKN3-specific inhibitors specifically into CCA cells.

CONCLUSION

It has been shown for the first time by this study that CDKN3 is upregulated in patient-CCA tissues and that CDKN3 plays an oncogenic role in increasing CCA proliferation. Silencing of CDKN3 could be a novel strategy to synergize gemcitabine cytotoxicity and reduce gemcitabine-resistance in CCA.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at https://dx.doi.org/10.2306/scienceasia1513-1874.2024.108.

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Appendix A. Supplementary data

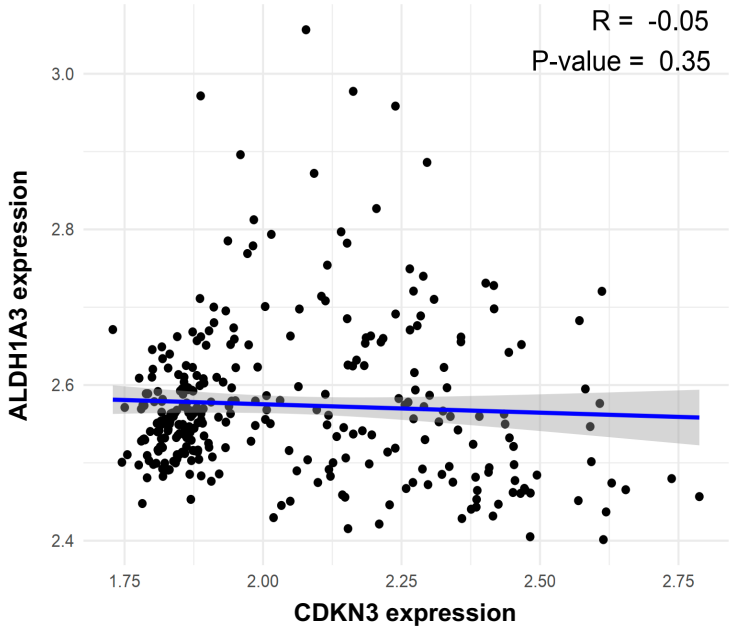

Fig. S1 Relation of CDKN3 and ALDH1A3 expression in CCA tissues. The scatter plot illustrates the relationship between the expression levels of CDKN3 and ALDH1A3 genes. The data were obtained from the GSE76297 dataset [18].

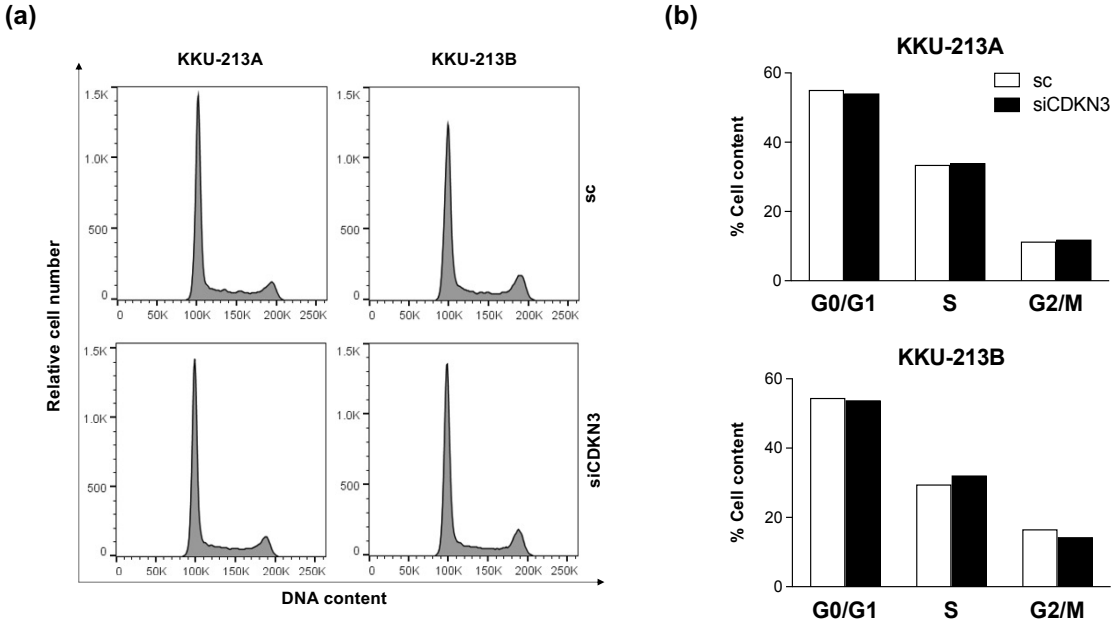

Fig. S2 Cell cycle distribution analysis of KKU-213A and KKU-213B cells treated with siCDKN3 or scramble siRNA using flow cytometry. (a), Cell cycle distribution pattern; and (b), percentages of the cells in each cell cycle phase.

Table S1 Univariate analysis on the association of CDKN3 expression and clinicopathologic characteristics of CCA patients (n = number of patients).

Variable		n	CDKN3 expression		
			Low (n)	High (n)	p
Age (years);	< 56	23	12	11	0.571
	$\geqslant 56$	25	11	14	
Sex;	Female	20	9	11	0.732
	Male	28	14	14	
Histological type;	Papillary	20	12	16	0.406
	Non papillary	28	11	9	
Tumor stage;	I–III	28	14	14	0.732
	IV	20	9	11	