Apoptosis-related protein-1 acts as a tumor suppressor in cholangiocarcinoma cells by inducing cell cycle arrest via downregulation of cyclin-dependent kinase subunits

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Abstract. Cholangiocarcinoma, a malignancy arising from the biliary tract, is associated with high mortality due to the late diagnosis and lack of effective therapeutic approaches. Our knowledge of the molecular alterations during the carcinogenesis of cholangiocarcinoma is limited. Previous study suggests that apoptosis-related protein-1 (Apr-1) is involved in cancer cell proliferation and survival. In the present study, we first detected the expression pattern of Apr-1 in human cholangiocarcinoma tissues and the effects of forced Apr-1 expression on cell proliferation and cell cycle progression. Cell cycle gene array analysis was used to identify downstream molecules that were regulated by Apr-1, and their expression levels were further evaluated in human cholangiocarcinoma tissues. We showed that Apr-1 expression was downregulated in human cholangiocarcinoma tissues. Forced expression of Apr-1 inhibited cell proliferation of cholangiocarcinoma cell line QBC939 and induced G2/M phase arrest. Downregulation of cell cycle-related genes cyclin-dependent kinase (Cdk) 2, and cyclin-dependent kinase subunits (Cks) 1 and 2 was involved in Apr-1-induced cell cycle arrest. Furthermore, we found that Cdk2 and Cks1/2 expression levels were elevated in human cholangiocarcinoma tissues. Taken together, our data showed that Apr-1 plays a crucial role in cell proliferation by controlling cell cycle progression, implying a tumor-suppressor function of Apr-1 in cholangiocarcinoma carcinogenesis. Thus, the present study provides a rationale to further study the underlying mechanisms of Apr-1 downregulation in cholangiocarcinoma for exploring potential diagnostic and therapeutic targets.

Introduction

Cholangiocarcinoma is defined as an epithelial tumor with features of cholangiocyte differentiation. According to the anatomic location, it is categorized as either intrahepatic or extrahepatic cholangiocarcinoma. Although cholangiocarcinoma accounts for 3% of all gastrointestinal tumors, it is the most common biliary malignancy and the second most common hepatic malignancy after hepatocellular carcinoma (1). The overall survival rate of cholangiocarcinoma of less than 5% at 5 years has not significantly changed over the past 3 decades (2). Surgical treatment is the preferred option for all subtypes. Yet, surgical intervention is limited due to the lack of effective markers for early diagnosis, thus only a small number of patients benefit from surgery. Furthermore, a high postoperative recurrence rate and low sensitivity to chemotherapeutics are critical factors which contribute to the poor prognosis of cholangiocarcinoma (3,4). Several risk factors including primary sclerosing cholangitis, liver fluke infestation and hepatolithiasis have been described. However, little is known concerning the mechanisms of carcinogenesis. Therefore, there is a dire need for improving our understanding of the biology of cholangiocarcinoma to develop effective early diagnostic and therapeutic options.

Apoptosis-related protein-1 (Apr-1), also known as melanoma-associated antigen (MAGE)-H1 and restin, was first cloned by our group from the apoptotic tumor cell line HL-60 when induced by all-trans retinoic acid (ATRA) (5). It belongs to the MAGE gene superfamily based on the bioinformatic analysis of its gene structure compared with other homologues in the GenBank (6). According to their expression patterns, MAGE genes can be divided into two groups. Type I MAGE genes are expressed in tumors of various histological origins, but are completely silent in normal tissues, with the exception of male germ cells and placenta; thus, the corresponding proteins represent attractive targets for cancer immunotherapy (6). On the contrary, type II MAGE genes are ubiquitously expressed...
in somatic cells, both in tumors and normal adult tissues, which suggests that they may play an important role in biological processes (7). Although the activation and expression of MAGEs were reported in various human cancers including cholangiocarcinoma, the physiological function of MAGEs remains largely unknown (8,9). However, the involvement of MAGEs in the regulation of cell cycle progression (10) and apoptosis (11,12) has been suggested. Thus, the identification of the mechanisms responsible for the biological functions of MAGEs may shed new light on the understanding of the cause and development of cholangiocarcinoma.

According to our previous study, the expression pattern of Apr-1 indicates it is a type II MAGE gene and is involved in cancer cell proliferation and survival (13,14). In the present study, we first examined the expression pattern of Apr-1 in human cholangiocarcinoma tissues, and then investigated the effects of induced overexpression of Apr-1 on cell growth and cell cycle regulation in human cholangiocarcinoma cell line QBC939. Furthermore, we analyzed the cell cycle-specific gene expression profile in QBC939 cells upon Apr-1 expression, as well as the expression of several key cell cycle regulatory proteins in human cholangiocarcinoma tissues. These studies facilitate a better understanding of the fundamental aspects of Apr-1, as a type II MAGE gene, in the tumorigenesis and tumor development of cholangiocarcinoma.

Materials and methods

Tissues. Four fresh-frozen cholangiocarcinoma samples and matched tumor-adjacent tissues obtained from Xijing Hospital (Fourth Military Medical University, Xi'an, Shaanxi Province, China) were collected and stored at -70°C. Formalin-fixed paraffin-embedded (FFPE) human cholangiocarcinoma tissue and tumor-adjacent tissue samples were collected from the Department of Pathology, Xijing Hospital. Ethical approval was obtained from the Xijing Hospital Ethics Committee.

Reagents. Anti-Apr-1 (anti-MAGE-H1) polyclonal antibody (HPA011324) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-Cdk2 (ab6538), anti-Cks1/2 (ab54643) and anti-β-actin (ab8227) rabbit polyclonal antibodies were purchased from Abcam (Shanghai, China). Restriction enzymes BamHI, XbaI, SalI, SacI, ExTaq polymerase, DNA marker DL2000, λDNA/EcoRI/HindIII marker were purchased from Takara (Dalian, China). LiposomeTM reagent, Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco-BRL (Gaithersburg, MD, USA). RNasin was purchased from Promega (Madison, WI, USA). G418 and TRIzol were from Invitrogen (Carlsbad, CA, USA). High-capacity cDNA reverse transcription kit was from Applied Biosystems (Carlsbad, CA, USA).

Plasmid. A BamHI site was introduced to the 5'-end and a SalI site was introduced to the 3'-end of the primers for cloning the open reading frame of Apr-1. The sequences were as follows: sense BamHI, 5'-TGGATCCgagacatgcctcggg-3'; antisense SalI, 5'-ACCGGTTCGACgtactcttaagggt-3'. The purified PCR products and pcDNA3.0 vector were cut by BamHI/SalI and BamHI/XhoI, respectively. The digested fragments were harvested and cloned into pcDNA3.0 between the same sites of SalI/XhoI to yield pcDNA3.0-Apr-1. The recombinant plasmid was confirmed by digestion of SalI/XhoI and sequencing.

Cell culture. Human cholangiocarcinoma QBC939 cells were stocked in our laboratory. Cells were cultured in DMEM containing 10% FBS, 50 IU/ml penicillin and 50 µg/ml gentamycin at 37°C under an atmosphere of 5% CO2. For gene transfection, QBC939 cells in optimal growth conditions were divided into 3 groups: the control (QBC939 cells), blank vector group (pcDNA3.0-transfected QBC939 cells) and experimental group (pcDNA3.0-Apr-1-transfected QBC939 cells). Transfection procedures were performed according to the manufacturer's instructions. The resistant cells were screened by G418. G418-resistant clones were obtained after a 2-week selection.

Quantitative RT-PCR. Total RNA from the frozen tissues or QBC939 cells was extracted using TRIzol following the manufacturer's instructions. Reverse transcription reactions were conducted using a high-capacity cDNA reverse transcription kit. All primers were optimized for amplification under reaction conditions as follows: 95°C for 10 min, followed by 30 cycles of 95°C for 15 sec and 60°C for 1 min. Melt curve analysis was performed for all samples after completion of the amplification protocol. β-actin was used as the housekeeping gene expression control. Listed below are the primer sequences used for quantitative PCR: Cdk2 forward, 5'-TCTCGCCA TTTCATCGGGTC-3' and Cdk2 reverse, 5'-ATTTGCGACG CAGGAGAGATT-3'; Cks1 forward, 5'-AGCCACCCGGATCTATGT-3' and Cks1 reverse, 5'-TCTCAGAAGCCAAG ATTCCT-3'; Cks2 forward, 5'-TCTCGTGACCTCTCGTT T-3' and Cks2 reverse, 5'-TGGACACCAAATCTCTCT CCA-3'.

MTT cell proliferation assay. Each group of QBC939 cells was seeded at 1x104 cells/well into a 96-well plate. For cell growth analysis, 20 µl freshly made MTT (5 mg/ml) was added into each well and incubated for 4 h at 37°C. Then, cell culture media were removed and replaced with 150 µl of dimethylsulfoxide (DMSO) for a further 10-min incubation with gentle shaking until the crystals were dissolved. The optical density (OD) value of each well was measured using a microculture plate reader (Coulter American) with a test wavelength of 490 nm. Three duplicate wells for each group were measured per day.

Cell cycle analysis. The floating and adherent cells were harvested and washed twice with phosphate-buffered saline (PBS), then re-suspended in 300 µl PBS and fixed by adding 700 µl cold ethanol in 70% ethanol at 4°C overnight. After washing twice with PBS, 100 µl of fixed cells (~1x10^6 cells) were stained with 300 µl propidium iodide (PI) for 15 min. Cell cycle analysis by flow cytometry was performed. The percentages of cells at the G1, G2 and S phases were measured.

Cell cycle gene expression array analysis. To determine the expression of cell cycle-specific genes, a GEArray Q series human cell cycle gene array kit (SuperArray Biosciences, Frederick, MD, USA) was used. Total RNA was isolated from...
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QBC939 cells transfected for 48 h in 100-mm dishes with 10.0 µg of either empty vector pcDNA3.0 or pcDNA3.0-Apr-1. Both RNA samples were reverse-transcribed to produce 32P-labeled probes following the manufacturer's protocol. Cell cycle-specific genes in the nylon membranes were hybridized to heat denatured radiolabeled probes at 55˚C for 16 h in a hybridization buffer provided by the manufacturer. After washing twice in 2X SSC/1% SDS, followed by two additional washes in 0.1X SSC/1% SDS at 55˚C for 15 min each, the membranes were exposed to X-ray film. The spots were detected by autoradiography, and the intensities of the corresponding spots in the two membranes were compared for two RNA populations used to generate the probes.

Western blotting. Immunoblot analysis was performed according to standard procedures using the following antibodies and dilutions: anti-Apr-1 1:500, anti-Cdk2 1:1,000, anti-Cks1/2 1:1,000, and anti-β-actin 1:1,000. Equal amounts of protein from the cells and tissues were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Shanghai, China). The membranes were blocked with milk, and primary antibody incubations were performed at room temperature for 2 h. Secondary antibody HRP-conjugated anti-rabbit (1:5,000) was used and signals were detected with SuperSignal West Pico Substrate (Thermo Scientific, Shanghai, China). The visualization of bands was performed by exposure to high-performance autoradiography film.

Immunohistochemistry. Paraffin-embedded tissue sections on microscopic slides were processed through a graded series of alcohols and rehydrated in distilled water. Heat-induced antigen retrieval was performed by citrate buffer (10 mmol/l concentration, pH 6.0), and standard indirect biotin-avidin immunohistochemical analysis was performed as previously described (15,16).

Statistical analysis. The data are presented as mean ± SEM. Statistical analysis was performed using SPSS 21.0. Statistical evaluation of the data was performed by two-way analysis of variance (ANOVA) or unpaired t-test (two groups). A value of p<0.05 was considered to indicate a statistically significant result.

Results

Apr-1 expression is reduced in human cholangiocarcinoma tissues. We performed western blotting and qRT-PCR assay to evaluate the Apr-1 expression in pooled samples of 4 human intrahepatic cholangiocarcinoma and paired tumor-adjacent hepatic tissues. The results showed that Apr-1 expression was significant lower in the cholangiocarcinoma tissues than that in the tumor-adjacent hepatic tissues (Fig. 1A and B). We next examined Apr-1 expression in 6 pathologically graded (moderate to well differentiated) human cholangiocarcinoma FFPE samples by immunohistochemistry (IHC). All normal or tumor-adjacent bile ducts exhibited medium to strong positive
staining of Apr-1 in both the cytoplasm and nuclei (Fig. 1C). In contrast, Apr-1 protein was undetectable in most of the cholangiocarcinoma tissues. Only rare carcinoma cells showed very weak trace nuclear expression of Apr-1 (Fig. 1D). We did not find any Apr-1 expression in the samples derived from human cholangiocarcinoma cell line QBC939 (data not shown). Therefore, it appears that Apr-1 expression is essential for normal cell function and is downregulated during cholangiocarcinoma development.

Apr-1 inhibits cell proliferation of QBC939 cells by inducing G2/M phase arrest. Given the fact that QBC939 cells do not express Apr-1, we sought to investigate the effects of induced overexpression of Apr-1 on cholangiocarcinoma cell proliferation and survival. QBC939 cells were transfected with pcDNA3.0-Apr-1, and stable cells were screened by G418. MTT assay was carried out to analyze the viability of the QBC939 cells with Apr-1 expression. The results showed that the growth of the QBC939-pcDNA3.0-Apr-1 cells was significantly inhibited (p<0.001, by two-way ANOVA) (Fig. 2A). Subsequently, we analyzed changes in the cell cycle distribution in the QBC939, QBC939-pcDNA3.0, and QBC939-pcDNA3.0-Apr-1 cells. The results demonstrated that QBC939-pcDNA3.0-Apr-1 cells were promoted to enter into the following phase from the G1 phase, which resulted in 4.2% more cells arrested in the G2/M phase, coinciding with 2.5% more cells accumulated in the S phase when compared with the QBC939-pcDNA3.0 group (Fig. 2B). Meanwhile, cell death was not observed in these three cell lines. These data indicate that Apr-1 may play a role in cholangiocarcinoma cell proliferation by negatively regulating the cell cycle at the G2/M point.

Cdk2, Cks1 and Cks2 are involved in Apr-1-induced cell cycle arrest. Based on the above data, we further investigated whether the transcription levels of cell cycle-regulatory genes are affected by expression of Apr-1. Cell cycle-specific gene expression array was used to determine the differences in gene expression between the QBC939-pcDNA3.0-Apr-1 and QBC939-pcDNA3.0 cells. The results showed that 23 cell cycle-specific genes including several cyclins and cyclin-dependent kinases (Cdks) were downregulated >2-fold in the QBC939-pcDNA3.0-Apr-1 cells compared with the levels in the QBC939-pcDNA3.0 group (Fig. 3A and Table I). There were also two genes, S phase kinase-associated protein 2 (Skp2) and ubiquitin-activating enzyme (UBE1) which are ubiquitin signaling pathway factors associated with the cell cycle, that were found to be upregulated upon Apr-1 expression (Table I). Notably, Cdk1 and Cdk2, cyclin-dependent kinase catalytic subunits that are important regulators of cell cycle transition between different cell cycle stages, were downregulated >4-fold.
Moreover, the expression levels of Cdk-interacting protein Cks1 and cyclin-dependent kinase subunit (Cks2), which are tightly associated with Cdks and mitotic entry, were decreased ~3-fold upon Apr-1 expression in the QBC939 cells.

Figure 3. Apr-1 expression in QBC939 cells regulates transcription of cell cycle-related genes Cdk2, Cks1 and Cks2. (A) Specific-cell cycle cDNA microarray analysis showed a discrepancy in gene expression pattern between QBC939-pcDNA3.0 and QBC939-pcDNA3.0-Apr-1 cells. (B) Western blot assay for Cdk2 and Cks1/2 expression in QBC939-pcDNA3.0 and QBC939-pcDNA3.0-Apr-1 cells. (C) Relative levels of Cdk2, Cks1 and Cks2 transcripts in the QBC939-pcDNA3.0 and QBC939-pcDNA3.0-Apr-1 cells as measured by qRT-PCR 24 h after transfection. Data represent the mean ± SEM. The statistical difference was determined by two-sided Student's t-test. *p<0.05.

Figure 4. Expression of cell cycle-related proteins Cdk2 and Cks1/2 in human cholangiocarcinoma tissues. (A) Western blotting assay for Cdk2 and Cks1/2 expression in human cholangiocarcinoma tissues. (B) Relative levels of Cdk2, Cks1 and Cks2 transcripts in normal tumor-adjacent hepatic (N) and cholangiocarcinoma tissues (Ch) as measured by qRT-PCR. Data represent the mean ± SEM. The statistical difference was determined by two-sided Student's t-test. *p<0.05.
To confirm the negative-regulatory effects of Apr-1 on Cdk2, Cks1 and Cks2 expression, we used western blotting and qRT-PCR assay to identify the changes in expression of these genes in the pcDNA3.0-Apr-1-transfected QBC939 cells. As shown in Fig. 3B and C, pcDNA3.0-Apr-1 transfection inhibited the expression of Cdk2 and Cks1/2 in the QBC939 cells at both the protein and mRNA levels. The results indicated that expression of Apr-1 induces G2/M phase arrest of cholangiocarcinoma cells potentially by inhibiting the transcription of Cdk2, Cks1 and Cks2.

Table I. Apr-1 regulates transcription of cell cycle-related genes in QBC939 cells (downregulated or upregulated >2-fold).

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<th>Description</th>
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Apr-1, apoptosis related protein-1.

To confirm the negative-regulatory effects of Apr-1 on Cdk2, Cks1 and Cks2 expression, we used western blotting and qRT-PCR assay to identify the changes in expression of these genes in the pcDNA3.0-Apr-1-transfected QBC939 cells. As shown in Fig. 3B and C, pcDNA3.0-Apr-1 transfection inhibited the expression of Cdk2 and Cks1/2 in the QBC939 cells at both the protein and mRNA levels. The results indicated that expression of Apr-1 induces G2/M phase arrest of cholangiocarcinoma cells potentially by inhibiting the transcription of Cdk2, Cks1 and Cks2.

Cdk2, Cks1 and Cks2 expression levels are elevated in human cholangiocarcinoma tissues. To further characterize the correlation between Apr-1 and Cdk2, Cks1 and Cks2 expression in cholangiocarcinoma, we detected Cdk2, Cks1 and Cks2 protein and mRNA levels in tumor and tumor-adjacent hepatic tissues. Western blotting showed that Cdk2 and Cks1/2 were accumulated in the samples of cholangiocarcinoma tissues compared with matched tumor-adjacent hepatic tissues. These data suggest that Apr-1 expression, which is reduced in cholangiocarcinoma, is negatively correlated with Cdk2, Cks1 and Cks2 expression.

**Discussion**

Cholangiocarcinoma is a highly malignant cancer with a poor prognosis. Abnormalities in various signaling cascades, molecules and genetic mutations are involved in the pathogenesis of cholangiocarcinoma. Recurrent mutations including KRAS, BRAF, TP53, Smad and p16 (INK4a) are characteristic of cholangiocarcinoma. KRAS and BRAF mutant cholangiocarcinomas have been associated with a worse long-term survival. Meanwhile, disruption of the RAF/MEK/MAPK pathway by RAS or BRAF mutation has been found in >60% of cholangiocarcinomas, indicating that these pathways are important in cholangiocarcinoma carcinogenesis (17). Wnt signaling also plays an important role in cholangiocarcinoma carcinogenesis through activation of downstream target genes such as cyclin D1 and c-myc (18,19). Although extensive efforts have been made to explore the transformation mechanism of cholangiocarcinoma in the past decades, detailed molecular and cellular mechanisms remain unclear. More comprehensive understanding of the mechanisms involved in the pathogenesis of cholangiocarcinoma is critical for the development of effective therapies.

Apr-1 belongs to the MAGE superfamily for which over 30 members have been identified. Although the first MAGE family gene was discovered in 1991, MAGEs have been well
studied for >20 years in melanomas (20), yet their functions still remain unclear.

Based on our previous study, Apr-1 protein was found to be localized in the nucleus and is believed to be an apoptosis-related gene since its transcripts were upregulated during all-trans-retinoic acid-induced apoptosis in human promyelocytic leukaemia cells (5,14). In situ hybridization assay on tissue microarrays showed that Apr-1 is expressed in esophageal carcinoma, normal hepatic tissue and hepatic tissue adjacent to hepatocellular carcinoma, but is absent in normal esophageal mucosa and hepatocellular carcinoma (data not shown). The expression pattern suggests that Apr-1 is a type II MAGE gene. Recently, type II MAGE proteins are under increasing attention due to their roles in the regulation of cell cycle progression and apoptosis.

In the present study, we found that Apr-1 triggered cell cycle arrest in QBC939 cells, QBC939-pcDNA3.0-Apr-1 cells showed a decrease in the G1 phase and were dominantly arrested in the G2/M phase after Apr-1 overexpression. Using gene expression array, we identified that Apr-1 induced G2/M phase arrest of QBC939 cells via a mechanism mediated by downregulation of the cell cycle checkpoint-related genes, in which Cdk2, Cks1 and Cks2 expression levels played critical roles during this course.

Cdns are a family of protein kinases that drive the cell cycle progression through their periodic activation. Cyclin-Cdk complexes phosphorylate specific substrates in a particular cell cycle phase (21). The cyclin B-Cdk1 complex is vital for entering mitosis (M phase), while Cdk2 is involved with cyclin A and E and activated from late G1 until the onset of mitosis (22). Moreover, deregulation of Cdk2 can result in DNA damage accumulation and loss of DNA damage checkpoint control (23-25). In the present study, we found that Cdk1 and Cdk2 were inhibited by Apr-1 expression. Thus suggests that Apr-1 induces cholangiocarcinoma cell cycle arrest in the G2 phase by impairing the expression of Cdk1 and Cdk2.

Given their essential role in cell cycle progression, Cdk1 and Cdk2 are highly regulated by, among others, cyclin-dependent-kinase subunit Cks1 and Cks2. The human cyclin kinase subunit family consists of two well-conserved members, Cks1 and Cks2, both of which were identified based on the sequence homology to yeast suc1 and Cks1 (also named Cdc28 kinase subunit 1) that are essential for cell cycle control (26). Accumulative evidence shows that the two Cks members have distinct regulatory functions in mammalian cells although 81% of protein products are identical and share one or more redundant functions in both humans and mice (27). Cks1 can specifically regulate cell cycle G1/S transition by promoting the process of SCF<sup>Skp2</sup>-mediated ubiquitination and p27<sup>Kip1</sup> (a Cdk2 inhibitor) degradation (28,29). Therefore, cells lacking Cks1 have elevated p27 and reduced Cdk2 activity (29,30). Cks2 has been shown to be essential for the first metaphase/anaphase transition in mammalian meiosis; however, its function is not clearly identified in the cell cycle (23,24,31,32). Recent study has shown that overexpression of Cks1 or Cks2 can maintain Cdk2 in an active state (33). Therefore, in the present study, decreased Cdk2 level upon Apr-1 expression could directly result from Apr-1 regulation and/or be mediated by Cks1 and Cks2 inhibition.

Numerous studies report abnormal Cks1/2 expression in various malignant tumors including hepatocellular carcinoma, bladder, gastric and breast cancer, and meningioma (34-38). However, the mechanistic link between Cks protein deregulation and oncogenesis remains to be elucidated. Liberal et al found that human mammary epithelial cells with Cks1 or Cks2 overexpression became resistant to DNA damage response mediated by cell cycle checkpoint that was triggered by oncoproteins, thus, allowing cells to continue proliferating even under replicative stress (33). More recently, Cks2 was found to be significantly elevated in cholangiocarcinoma tissues and its downregulation inhibited cholangiocarcinoma cell proliferation in vitro and in vivo; particularly, Cks2 knockdown induced cholangiocarcinoma cell cycle arrest in the G2/M phase by facilitating cell apoptosis, which suggests that Cks2 may serve as an independent prognostic factor in patients with cholangiocarcinoma (39). Consistently, we found that Cks1 and Cks2 were accumulated in human cholangiocarcinoma samples. Most importantly, we demonstrated that Apr-1 expression was downregulated in cholangiocarcinoma and induced overexpression of Apr-1 in cholangiocarcinoma cells inhibited cell proliferation, mechanistically mediated by Cks1 and Cks2 deficiency, indicating that Apr-1 can regulate the expression of Cks1 and Cks2 in cholangiocarcinoma. Two cell cycle-related genes, Skp2 and UBE1, showed >2-fold increase at the transcription level after overexpression of Apr-1. Skp2 is a member of the F-box family of substrate-recognition subunits of SCF ubiquitin-protein ligase complexes that has been implicated in ubiquitin-mediated degradation (40); UBE1, also known as an E1 enzyme, catalyzes the first step in the ubiquitination reaction (41). Both Skp2 and UBE1 function to regulate other cell cycle molecules by ubiquitination. It has been recognized that Skp2 can interact with Cks1 to degrade p27<sup>Kip1</sup> protein, which serves as a negative cell cycle regulator exemplifying a class of tumor suppressors that controls cell cycle progression (42). Recent findings indicate that high expression of Skp2 is associated with aggressiveness and a poor prognosis in a large variety of cancers, including cholangiocarcinoma (43). The present study did not address whether there is a feedback loop leading to upregulation of Skp2 upon Apr-1-induced cell cycle arrest. Additional studies are required to clarify the regulatory mechanisms involved in elevated Skp2 and UBE1 transcription induced by Apr-1 overexpression.

In conclusion, our data strongly suggest that Apr-1 has a tumor-suppressor function in cholangiocarcinoma cells, mechanistically by inducing cell cycle arrest through regulating a series of cell cycle regulators. Thus, our study lays a foundation for further investigation of the underlying mechanisms of Apr-1 downregulation during the development and progression of cholangiocarcinoma in order to explore potential therapeutic targets for cholangiocarcinoma treatment.

Acknowledgements

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References


