Aspirin inhibits cholangiocarcinoma cell proliferation via cell cycle arrest in vitro and in vivo

TINGTING SHI¹, JIAN GONG², KOJI FUJITA¹, NORIKO NISHIYAMA¹, HISAKAZU IWAMA³, SHI LIU¹, MAI NAKAHARA¹, HIROHITO YONEYAMA¹, ASAHIRO MORISHITA¹, TAKAKO NONURA¹, HIDEKI KOBARA¹, KEIICHI OKANO³, YASUYUKI SUZUKI⁴ and TSUTOMU MASAKI¹

¹Department of Gastroenterology and Neurology, Faculty of Medicine, Kagawa University, Kida, Kagawa 761-0793, Japan; ²Department of Gastroenterology, The First Affiliated Hospital of Dalian Medical University, Dalian, Liaoning 116011, P.R. China; ³Life Science Research Center, Kagawa University; ⁴Department of Digestive Surgery, Faculty of Medicine, Kagawa University, Kida, Kagawa 761-0793, Japan

Received June 24, 2020; Accepted October 23, 2020

DOI: 10.3892/ijo.2020.5165

Abstract. Cholangiocarcinoma is the most common biliary duct malignancy and the second most common primary liver cancer, accounting for 10-20% of hepatic malignancies. With high mortality and poor prognosis, the 5-year survival rate of cholangiocarcinoma is only 10%. A previous study demonstrated a significant association between aspirin use and a decreased risk of cholangiocarcinoma. However, the effect of aspirin on cholangiocarcinoma remains unknown. Therefore, the aim of the present study was to investigate the effects of aspirin on cholangiocarcinoma in vitro and in vivo. Three cholangiocarcinoma cell lines were used to analyze the effect of aspirin on cell proliferation, cell cycle progression, apoptosis, and the regulation of microRNAs. MicroRNAs are known to regulate the development and progression of various types of cancer. An HuCCT-1 xenograft model was used for the in vivo study. It was determined that aspirin inhibited the proliferation of human cholangiocarcinoma cells (except TKKK cells). Aspirin induced cell cycle arrest in the G0/G1 phase and regulated cell-cycle related proteins in cholangiocarcinoma cells (HuCCT-1 cells) but did not induce apoptosis. The expression of miR-340-5p was significantly upregulated after treatment, and overexpression of miR-340-5p inhibited the proliferation of HuCCT-1 cells and decreased the levels of cyclin D1. TKKK cells had low miR-340-5p expression, which may explain why aspirin had no effect on their proliferation. In vivo, aspirin reduced the growth of xenografted tumors. In conclusion, the present study indicated that aspirin partially inhibited cholangiocarcinoma cell proliferation and tumor growth by inducing G0/G1 phase cell cycle arrest, potentially through the miR-340-5p/cyclin D1 axis.

Introduction

Cholangiocarcinoma (CCA) is the most common biliary duct malignancy and the second most common primary liver cancer, accounting for 10-20% of all primary hepatic malignancies (1). CCA arises from bile duct epithelial cells, and there are three subtypes based on anatomical location: Intrahepatic (iCCA), perihilar (pCCA), and distal (dCCA). The etiology of CCA includes lithiasis (2), primary sclerosing cholangitis (3), parasitic infection (4), congenital abnormalities (5), chronic liver disease (6), cirrhosis (6), metabolic abnormalities (7), and lifestyle (7). The incidence of CCA appears to be increasing (8); the rates of CCA in North America, Japan, and Australia have been rising over the past two decades (9). Although surgery is the preferred treatment, the 5-year postoperative survival rate is markedly low. Chemotherapy can be used for inoperable cases; however, the highly desmoplastic nature, rich tumor microenvironment, and profound genetic heterogeneity all contribute to iCCA therapeutic resistance (9).

The nonsteroidal drug aspirin is an anti-inflammatory and anticoagulation agent used to prevent and reduce the risk of cardiovascular events. According to some clinical analyses, long-term use is also associated with a reduction in cancer risk, including colon (10), breast (11), and hepatocellular carcinoma (12). Aspirin use also has a significant inverse association with the development of all three CCA subtypes and an approximately 3-fold reduction in CCA risk (13). Numerous anticancer mechanisms for aspirin have been...
identified, such as inhibition of cyclooxygenase (14), activating key molecular targets in the AMPK, mTOR, STAT3, and NF-xB pathways (15), decreasing the levels of reactive oxygen species and glucose consumption (16), inducing autophagy via JNK/p-Bcl2/Becn1-1, AMPK/mTOR, and GSK-3 signaling (17), inducing apoptosis and mitochondrial dysfunction by increasing oxidative stress (18), and changing the tumor microenvironment by affecting platelets (19,20). However, the effect of aspirin on CCA remains unknown. Elucidation of the mechanism of aspirin in CCA could contribute to the development of new therapeutic agents in the future.

The purpose of the present study was to determine the antitumor effects of aspirin in CCA and identify the key molecular targets and microRNAs (miRNAs) associated with this effect.

Materials and methods

Chemicals. Aspirin was obtained from Wako Pure Chemical Industries, Ltd. The prepared solution was diluted in cell culture medium as per the requirement of cells and fresh pH 7.2 to 7.5, within the range suitable for cell growth was used.

Cell lines and cell culture. Human CCA cell lines (HuCCT-1, RBE, and TKKK) were obtained from the Japanese Research Resources Bank. HuCCT-1 and RBE cells were grown in RPMI-1640 media (Gibco-Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS) (product no. 557-30355; FUJIFILM Wako Pure Chemical Industries, Ltd.) and penicillin/streptomycin (100 mg/l; Invitrogen; Thermo Fisher Scientific, Inc.). TKKK cells were maintained in DMEM (Gibco-Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and penicillin/streptomycin. All cell lines were grown in a humidified incubator at 5% CO₂ and 37°C.

Cell viability assay. Cell viability assays were performed using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc.) according to the manufacturer’s instructions. Briefly, cells (5,000 cells/well) were seeded into 96-well plates and treated with 2.5 mmol/l aspirin for 48 h. The cells were lysed in polyoxyethylene octyl phenyl ether (Pure Chemical Industries, Ltd.) followed by incubation with 10 µl of PBS with 10 µl RNase A (250 µg/ml) and 10 µl propidium iodide (PI) stain (100 µg/ml) and incubated at room temperature in the dark for 30 min. Flow cytometry (FCM) was performed to compare the proportion of aspirin-treated and control cells in each phase of the cell cycle. FCM was performed using a Cytomix FC 500 flow cytometer (Beckman Coulter, Inc.) with an argon laser (488 nm), and the percentages of cells were analyzed using the Caluza software version v2.1 (Beckman Coulter, Inc.). The experiments were repeated thrice.

Apoptosis analysis. Aspirin-mediated apoptosis was analyzed using Annexin V-FITC Early Apoptosis Detection kit (Cell Signaling Technology, Inc.). HuCCT-1 cells (1.0×10⁶ cells/100-mm dish) were treated with 2.5 mmol/l aspirin or without for 48 h. Apoptotic and necrotic cells were analyzed by double staining with FITC-conjugated Annexin V and PI per the manufacturer’s instructions. FCM was conducted using a Cytomix FC 500 flow cytometer with an argon laser (488 nm) to compare the proportion of apoptotic cells in the aspirin-treated and control groups, and data were analyzed using the Caluza software version v2.1. The experiments were repeated thrice.

Western blot analysis. HuCCT-1 and TKKK cells were seeded (1.0×10⁶ cells/100-mm dish) and treated with 2.5 mmol/l aspirin for 24 or 48 h. The cells were lysed with PRO-PREP complete protease inhibitor mixture (iNTRON Biotechnology, Korea). Supernatants were collected, and the protein concentrations were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). Protein aliquots (10 µg) were resuspended in sample buffer and separated on 12% Tris-glycine gradient gels via sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The resolved proteins were transferred to a nitrocellulose membrane and blocked with a blocking buffer containing 2% skimmed milk (GE Healthcare) in TBST with 0.1% Tween 20. The following primary antibodies in 2% skimmed milk were used: Cyclin D1 (SP4) (1:2,000 dilution; cat. no. MA5-14512), retinoblastoma (Rb) (1:1,000 dilution; cat. no. MA1-34070), and cyclin E (HE-12) (1:1,000 dilution; MS-870-P1) were obtained from Thermo Fisher Scientific, Inc.; phosphorilated Rb (pS780) (1:1,000 dilution; cat. no. 558554) was obtained from BD Biosciences; Cdk2...
The primer sequences are as follows:

- miR-340-5p forward, 5'-GCTTGGGGA TCG-3'; U6 forward, 5'-GCTTGGGCA GCACATACTAAAAT-3' and reverse, 5'-CGCTTCA CGAATTGCCGTGCAT-3'.

Bioinformatics analysis used for prediction of the target genes of miR-340-5p and functional and network analyses. For bioinformatics analysis, miRDB database (www.mirdb.org) was used to predict the target genes of miR-340-5p and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were used for upregulated miRNAs depending on the Database for Annotation, Visualization, and Integrated Discovery (DAVID database) (22). miRNA regulatory network used mirPath v.3 (http://snf515788.vm.okeanos.grnet.gr) (23); miRgator v3.0 (http://mirgator.kobic.re.kr) (24) and miTarBase (http://miTarbase.cuhk.edu.cn/php/index.php) (25) online software to reveal the expression of miR-340-5p in related cancer and normal tissue and the relationship with the underlying target gene. The cut-off criterion was set as P<0.05.

Cell transfection. For transfection, 5×10^5 HuCCT-1 cells were seeded in 6-well plates with antibiotic-free medium 1 day before transfection to reach a confluence of 90% at the time of transfection. Cells were transfected with miR-340-5p mimic (50 nM) (sense, 5'-UUAAUAACGAGACAGAUAU-3' and antisense, 5'-UCAGUCUUCUUGUUAAATT-3'), inhibitor (50 nM) (5'-AAUGAGUCAUGUGUUAUA-3'), or negative control miRNA (50 nM) (mimic negative control 5'-UUGUACUACACAAAGUUACUG-3'; inhibitor negative control 5'-CAGUACUUUGUAGUACCA-3') (Life Technologies; Thermo Fisher Scientific, Inc.) using Lipofectamine RNAiMax Reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The transfection complex was prepared according to the manufacturer's instructions and added to the cells, and the plates were incubated in a humidified atmosphere with 5% CO2 at 37°C. Cells cultured with transfection reagent as untransfected control. The medium was replaced 6 h post-transfection. Cell samples were collected at 0 or 48 h after transfection for further analysis.

Colony formation assay. Cells were trypsinized for 5 min and resuspended at a density of 1×10^5/ml. Five hundred microliters were seeded into 6-well plates, and 1.5 ml RPMI-1640 medium containing 10% FBS was added to each well. The plates were incubated at 37°C at 5% CO2. The plates were incubated at 37°C at 5% CO2. Cells were incubated for 7 days, and the plates were incubated in a humidified atmosphere with 5% CO2 at 37°C. Cells cultured with transfection reagent as untransfected control. The medium was replaced 6 h post-transfection. Cell samples were collected at 0 or 48 h after transfection for further analysis.

Xenograft model analysis. The animal study was conducted in accordance with the guidelines set by the Committee on Experimental Animals of the Kagawa University. All experimental protocols were approved (approval no. 18674) by the Institutional Review Board of the Department of Laboratory Animal Science of Kagawa University (Kagawa, Japan). Thirty-five female athymic mice (BALB/c-nu/nu; 6 weeks old; 19-21 g) were purchased from Japan SLC (Shizuoka, Japan). The mice were maintained at 20-25°C with 30-60% humidity under a 12:12 h light/dark cycle, using a laminar airflow rack.
and had continuous free access to sterilized (γ-irradiated) food (CL-2; CLEA Japan, Inc.) and autoclaved water. Mice were subcutaneously inoculated with 1.5x10⁶ HuCCT-1 cells in the right flank. When the xenografts were palpable with an approximate diameter of 3 mm, we randomly assigned the animals to three groups of 10 animals each. These groups were treated with 60 mg/kg aspirin, 100 mg/kg aspirin, or vehicle (10% ethanol in PBS) by intraperitoneal injection every day. The tumor volume (mm³) was calculated as tumor length (mm) x tumor width (mm)²/2. For humane endpoints, if difficulty in feeding and/or intake of water; apparent poor physical condition; a rapid and non-recoverable weight loss (over 20% body weight); and/or a significant increase in tumor size was observed (tumor size more than 10% of body weight and/or tumor diameter more than 20 mm), the experiments were discontinued. Animals were euthanized through CO₂ euthanasia with 20% displacement of cage volume/min. Sacrifice was confirmed by observation of unconsciousness, absence of heartbeat and absence of breathing. All animals were sacrificed on day 28 of treatment. The experiments were performed from February 20, 2019 to March 28, 2019.

**Immunohistochemistry.** Immunohistochemistry was performed on tumor tissues obtained from the xenografted mice. We prepared 5-µm-thick sections from formalin-fixed (10% formalin at room temperature for 24 h), paraffin-embedded tissue blocks which were deparaffinized, rehydrated, and subjected to immunohistochemistry studies. Following a blocking step at room temperature for 30 min using a Vectastain Elite ABC kit (Vector Laboratories, Inc.), tissue sections were incubated with primary antibodies [Cyclin D1 (EP12); 1:200 dilution; cat. no. 241R-4, Sigma-Aldrich; Merck KGaA]. Sections were washed and incubated with anti-rabbit IgG Antibody (1:50 dilution; cat. no. BP-9100-50; Vector Laboratories) at room temperature for 1 h and then with a streptavidin-peroxidase solution. Color reactions involved the use of 3,3'-diaminobenzidine (DAB) with Mayer's hematoxylin counterstaining at room temperature for 10 sec. The specificity of immunostaining was evaluated using non-immune mouse IgG (1:50 dilution; cat. no. BP-9200-50; Vector Laboratories) at room temperature for 1 h as a negative control for the primary antibody. Sections were examined microscopically for specific staining, and nuclei with a brown color regardless of staining intensity were regarded as positive. Cyclin D1 positivity was calculated by calculating Pearson's centered correlation coefficient, and early apoptotic cells with aspirin treatment and without treatment were similar after 48 h (Fig. 3A). Additionally, there was a significant increase in the proportion of late apoptotic cells (upper right quadrant). The proportion of early apoptotic cells with aspirin treatment and without treatment were similar after 48 h (Fig. 3A). Western blot results indicated that treatment with 2.5 mmol/l aspirin for 24 or 48 h, and untreated cells were used as the control. Following aspirin treatment, the population in the G1/G0 phase significantly increased, whereas cells in the S phase decreased (Fig. 2A and B). Western blot results indicated that treatment with aspirin for 48 h significantly modulated cyclin D1, a key protein expressed in the early G1 phase. Cyclin D1 is a key regulator of the cell cycle and is involved in the transition from the G1 phase to the S phase (26). The levels of phosphorylated Rb decreased with aspirin treatment, suggesting that the treated cells were in G1 arrest (Fig. 2C).

**Aspirin does not induce cell apoptosis in HuCCT-1 cells.** To determine whether aspirin induced apoptosis in HuCCT-1 cells, FCM was used to detect apoptotic cells after aspirin treatment. The different quadrants represent living cells (lower left quadrant), early apoptotic cells (lower right quadrant), and late apoptotic cells (upper right quadrant). The proportion of early apoptotic cells with aspirin treatment and without treatment were similar after 48 h (Fig. 3A). Additionally, there was no obvious difference in c-Crk-18 levels between treated and untreated cells after 24- or 48-h treatments (Fig. 3B).

**Aspirin affects miRNA expression in HuCCT-1 cells.** A customized microarray platform was used to analyze the expression of 2,555 miRNAs in aspirin-treated and control HuCCT-1 cells. Treatment with 2.5 mmol/l aspirin for 48 h induced the overexpression of eight miRNAs, whereas the expression of six miRNAs was decreased (Table 1; Fig. 4). Unsupervised hierarchical clustering analysis was conducted by calculating Pearson’s centered correlation coefficient, and the results indicated that differentially expressed miRNAs in aspirin-treated HuCCT-1 cells clustered together. Previous studies have indicated that miR-340-5p has tumor-suppressive properties (27,28), and miR-340-5p exhibited functions in different parts in the KEGG pathway analyses of upregulated miRNAs, which included ‘prion diseases’, ‘microRNA in cancer’, ‘proteoglycans in cancer’, and ‘signaling pathways regulating pluripotency of stem cells’ as well as others (Fig. 5A). Although the expression of miR-340-5p was not clearly revealed in CCA, it was decreased in most types of cancer (Fig. 5B). The miRNA regulatory network and miRDB database predicted...
Figure 1. Aspirin inhibits the proliferation of some CCA cells. All cell lines were treated with 0, 2.5, 5, or 10 mmol/l aspirin for 24 or 48 h. The data points represent the mean cell number from three independent cultures, and the error bars represent standard deviations. The antiproliferative effect was significantly higher in cells treated with aspirin treatment for 48 h than in control cells (0 mmol/l) as determined by two-way analysis of variance, except for TKKK cells. *P<0.01, vs. the control. CCA, cholangiocarcinoma.

Figure 2. Aspirin causes cell cycle arrest in HuCCT-1 cells. (A) HuCCT-1 cells treated with or without 2.5 mmol/l aspirin were analyzed by flow cytometry to estimate the proportion of cells in each phase of the cell cycle. (B) Graphical representation of the proportion of cells in each phase of the cell cycle. Aspirin blocked the cell cycle at the G0/G1 phase. **P<0.01, vs. the control. (C) Expression of cyclin D1, cyclin E, Cdk2, Rb, and pRb in HuCCT-1 cells 24 and 48 h after aspirin treatment. Rb, retinoblastoma; pRb, phosphorylated Rb.

Figure 3. Aspirin does not induce apoptosis in HuCCT-1 cells. (A) Aspirin treatment (2.5 mmol/l for 48 h) did not alter the proportion of early apoptotic HuCCT-1 cells as determined by flow cytometry. (B) The expression of caspase-cleaved cytokeratin 18 as determined using ELISA after 24 or 48 h of 2.5 mmol/l aspirin treatment.
that CCND1 may be a target of miR-340-5p (Fig. 5C and D). Thus, it was selected for further study, and RT-qPCR indicated that miR-340-5p levels were significantly upregulated in the aspirin-treated cells compared to the untreated cells (Fig. 4).

Numerous other studies have revealed the effects of miRNAs on CCA in recent years (Table II) (29-47).

Table I. Statistical results and chromosomal locations of microRNAs in HuCCT-1 cells.

| miRNA             | Fold change (Treated/Untreated) | P-value | Chromosomal location |
|-------------------|---------------------------------|---------|---------------------|
| **A, Upregulated**|                                 |         |                     |
| hsa-miR-6504-3p   | 3.092                           | 0.021   | 16                  |
| hsa-miR-4266      | 2.701                           | 0.005   | 2q13                |
| hsa-miR-4708-3p   | 2.158                           | 0.028   | 14                  |
| hsa-miR-532-5p    | 2.055                           | 0.039   | X                   |
| hsa-miR-340-5p    | 2.029                           | 0.039   | 5                   |
| hsa-miR-203a-3p   | 1.552                           | 0.018   | 14                  |
| hsa-miR-1180-3p   | 1.547                           | 0.009   | 17                  |
| hsa-miR-146a-5p   | 1.522                           | 0.022   | 5                   |
| **B, Downregulated**|                                 |         |                     |
| hsa-miR-6744-3p   | 0.654                           | 0.003   | 11                  |
| hsa-miR-6752-3p   | 0.652                           | 0.000   | 11                  |
| hsa-miR-6859-5p   | 0.640                           | 0.041   |                     |
| hsa-miR-4669      | 0.636                           | 0.030   | 9q34.2              |
| hsa-miR-3607-5p   | 0.526                           | 0.045   |                     |
| hsa-miR-604       | 0.445                           | 0.011   | 10p11.23            |

Cells treated with aspirin that exhibited a fold change (FC)>1.5, FC<0.67, or a P-value <0.05 compared with untreated cells are presented.
Figure 5. Bioinformatics analysis and the predicted target gene of miR-340-5p. (A) The Kyoto Encyclopedia of Genes and Genomes pathway analyses of upregulated miRNAs. (B) Expression levels of miR-340-5p in related cancer and normal tissue. Compared with normal tissue, the expression of miR-340-5p was revealed to be decreased in most types of cancer. (C) miRNA regulatory network of miR-340-5p. The intensity of evidence between miR-340-5p with predicted target genes and relationships with associated miRNAs are presented in this network. (D) The seed sequence of human miR-340-5p was complementary to the 3'UTR of CCND1.
miR-340-5p inhibits the proliferation of HuCCT-1 cells and decreases the expression levels of cyclin D1. After transfection of miR-340-5p mimics, miR-340-5p expression was significantly increased in the HuCCT-1 cells (Fig. 6A). Transfection of miR-340-5p mimics decreased proliferation in HuCCT-1 cells (Fig. 6B). Moreover, overexpression of miR-340-5p decreased the levels of cyclin D1, whereas inhibition induced increased cyclin D1 levels (Fig. 6D). The colony formation assay indicated that overexpression of miR-340-5p decreased the cell proliferation ability of HuCCT-1 cells (Fig. 6C). Therefore, increasing the levels of miR-340-5p inhibited cyclin D1 expression and decreased the cell proliferation ability.
Figure 7. Comparison of the cell cycle and miR-340-5p in aspirin-nonresponsive TKKK cells and aspirin-responsive HuCCT1 cells. (A) Left image: TKKK cells treated with or without 2.5 mmol/l aspirin and analyzed by flow cytometry to estimate the proportion of cells in each phase of the cell cycle. Right image: Graphical representation of the proportion of cells in each phase of the cell cycle. (B) Expression of cyclin D1 in HuCCT-1 cells and TKKK cells after 48 h of aspirin treatment. (C) Relative quantification of miR-340-5p in cholangiocarcinoma cell lines.

Figure 8. Aspirin inhibits the growth of HuCCT-1 cell xenografts in nude mice. (A) The tumors were significantly smaller in aspirin-treated mice than in vehicle-treated mice. Each point represents the mean ± standard deviation of 10 animals. *P<0.01 by mixed analysis of variance. (B) Relative quantification of miR-340-5p in tumor tissue of the three groups of xenografted mice. (C) Left image: H&E-stained images of the xenografted tumor tissues between the aspirin-treated and control mice (scale bar, 100 µm). Immunohistochemical staining of cyclin D1 between the aspirin-treated and control mice (scale bar, 20 µm). Right image: Cyclin D1-positive cells in the aspirin-treated groups were reduced in number compared with the control group as determined by one-way analysis of variance. *P<0.05 and **P<0.01. H&E, hematoxylin and eosin.
Aspirin-nonresponsive TKKK cells express low levels of miR-340-5p. As the CCA cell line TKKK did not exhibit response to aspirin, the cell cycle progression in TKKK cells as compared to HuCCT-1 cells was assessed. Forty-eight hours of aspirin treatment in TKKK cells revealed no obvious difference in the proportion of cells in each phase of the cell cycle (Fig. 7A). In addition, the levels of cyclin D1 were not significantly altered (Fig. 7B). Relative quantification of miR-340-5p was assessed in all cell lines (Fig. 7C). Expression was lowest in TKKK cells, which may indicate that cell lines with high expression of miR-340-5p are more sensitive to cell cycle arrest with aspirin treatment.

Aspirin inhibits tumor proliferation in vivo. Based on the results obtained from in vitro studies, the effect of aspirin in an in vivo model of CCA was assessed. Nude mice were injected subcutaneously with HuCCT-1 cells followed by intraperitoneal injection of aspirin. The present results revealed that tumor growth was significantly inhibited in mice treated with aspirin compared to untreated mice (P<0.05) (Fig. 8A). No mice succumbed during the observation period. Expression levels of miR-340-5p in tumor tissue were not significantly different, although the RQ was slightly increased in the two aspirin-treated groups (Fig. 8B). H&E-stained images of the xenografted tumor tissues revealed no significant pathological differences between the aspirin-treated and control mice. Immunohistochemical staining of cyclin D1 indicated that cyclin D1-positive cells in the aspirin-treated groups were reduced in number compared with the control group (Fig. 8C).

Discussion

CCA is an aggressive cancer with high mortality and poor prognosis that accounts for approximately 3% of gastrointestinal malignancies (8). The 5-year survival rate of CCA is only 10%, and the median survival is 24 months (48). Initial analysis revealed that aspirin use was associated with a reduced iCCA risk in men (HR=0.64, 95% CI=0.42-0.98) (49). Subsequent analysis has revealed that aspirin use has a significant inverse association with the development of all three CCA subtypes and leads to an approximately 3-fold reduction in CCA risk (13). This is thought to be due to the anti-inflammatory effect of aspirin. In the present study, we further elucidated the mechanism of the effect of aspirin on CCA. To the best of our knowledge, the present study is the first study revealing that aspirin inhibits the proliferation of CCA cells in vivo.

The anti-inflammatory dose of aspirin varies from 0.5-2.5 mM (50); therefore, relying on data from the anti-proliferation assay, 2.5 mM was selected as the concentration of aspirin, which does not have off-target cytotoxicity. It was observed that aspirin inhibited the proliferation of CCA cells (HuCCT-1 and RBE) and induced cell cycle arrest (HuCCT-1) at the G0/G1 phase, which was correlated with a marked decrease in the expression of cyclin D1. Aspirin also decreased the phosphorylation of Rb. The expression of cell cycle-related molecules is related to cancer progression and prognosis (51), and aspirin has been revealed to inhibit the expression of cyclin D1 in other types of cancer, such as oral squamous cell carcinoma (52) and glioblastoma multiforme (53). To determine whether aspirin induced apoptosis, HuCCT-1 cells were treated with 2.5 mM aspirin and analyzed using FCM; the levels of cCK18 were also measured. However, there was no evidence that aspirin induced apoptosis in HuCCT-1 cells. These data indicated that aspirin inhibited CCA cell proliferation mainly through cell cycle arrest. However, aspirin has also been revealed to induce apoptosis in numerous types of cancers (52-55); this discrepancy could be due to differences in the properties of different types of cancers.

miRNAs are short, noncoding, endogenous, single-stranded RNA molecules 19-25 nucleotides in length that regulate target gene expression (56). They are known to regulate the development and progression of various cancers (57). A miRNA expression array was used to identify the miRNAs associated with the antitumor effects of aspirin. miR-340-5p that was significantly upregulated in response to aspirin treatment in cells, was not significantly different in tumor tissue, although the relative quantification (RQ) was slightly increased in the two aspirin-treated groups. Recent studies have indicated that miR-340-5p inhibited non-small cell lung cancer cell growth and metastasis by targeting ZNF503 (58) and suppressed osteosarcoma development via targeting STAT3 (59). In the present study, overexpression of miR-340-5p inhibited proliferation in HuCCT-1 cells. In addition, overexpression of miR-340-5p decreased the levels of cyclin D1. The predicted sequence from the miRDB database and bioinformatics analysis indicated that cyclin D1 may be an miR-340-5p target, and miR-340-5p overexpression decreased cyclin D1 levels in cells; immunohistochemical staining indicated that cyclin D1-positive cells in aspirin-treated mice were reduced compared with control mice. Therefore, it is theorized that aspirin works partially through the miR-340-5p/cyclin D1 axis to inhibit HuCCT-1 cell proliferation.

In the present study, aspirin treatment had no effect on TKKK cells. To investigate the difference between TKKK cells and HuCCT-1 cells, FCM was used to analyze changes in the cell cycle with aspirin treatment. Aspirin did not induce G0/G1 arrest in TKKK cells, and there was no difference in the levels of cyclin D1 after 48 h of treatment. In addition, the relative levels of miR-340-5p were assessed in all cell lines, and TKKK cells had the lowest expression. Therefore, aspirin may not suppress proliferation in TKKK cells because it cannot utilize the miR-340-5p/cyclin D1 axis.

In the present in vivo model, aspirin inhibited the growth of subcutaneous CCA tumors in athymic nude mice. In accordance with the in vitro results and previous studies (23,60), in the in vivo experiment, the tumor volumes in both treatment groups (low-dosage and high-dosage) were significantly decreased compared with the tumor volume in the control group. However, there was no obvious difference in tumor volume between the low-dosage and high-dosage groups; long-term treatment may be required to see a difference based on dosage.

In conclusion, the present study indicated that aspirin inhibited cell proliferation and tumor growth in some CCA cell lines by inducing G0/G1 phase cell cycle arrest, and the underlying mechanism may partially be through the miR-340-5p/cyclin D1 axis to induce cell cycle arrest.
Acknowledgements

We thank Ms. Kayo Hirose, Ms. Keiko Fujikawa, Ms. Miwako Watanabe, Ms. Megumi Okamura, and Ms. Fuyuko Kokado at the Department of Gastroenterology and Neurology of Kagawa University (Kagawa, Japan) for their assistance.

Funding

No funding was received.

Availability of data and materials

All data supporting the conclusions of the present study have been documented in this study.

Authors' contributions

TS, AM, HK and TM conceived and designed the experiments. TS, HI, JG, NN, SL, MN, HY, TN, KO, YS and KF analyzed and interpreted the data. TS performed the experiments. TS wrote, reviewed and edited the manuscript. TM reviewed and edited the manuscript for important intellectual content. All authors reviewed, read and approved the final manuscript.

Ethics approval and consent to participate

All experimental protocols were approved (approval no. 18674) by the Institutional Review Board of the Department of Laboratory Animal Science of Kagawa University (Kagawa, Japan).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Patel T: Increasing incidence and mortality of primary intrahepatic cholangiocarcinoma in the United States. Hepatology 33: 1353-1357, 2001.
2. Xiao J, Zhu J, Liu Z, Wan R, Li Y and Xiao W: Role of surgical treatment for hepatolithiasis-associated intrahepatic cholangiocarcinoma: A retrospective study in a single institution. J Cancer Res Ther 13: 756-760, 2017.
3. Arbelaitz A, Azkargorta M, Krawczyk M, Santos-Laso A, Lapitz A, Perugorria MJ, Erico O, Gonzalez E, Jimenez-Agirre R, Lacasta A, et al.: Serum extracellular vesicles contain protein biomarkers for primary sclerosing cholangitis and cholangiocarcinoma. Hepatology 66: 1125-1137, 2017.
4. Kim TS, Pak JH, Kim JB and Bahk YY: Clonorchis sinensis, an oriental liver fluke, as a human biological agent of cholangiocarcinoma: A brief review. BMB Rep 49: 590-596, 2016.
5. Jang MH, Lee YJ and Kim H: Intrahepatic cholangiocarcinoma arising in Caroli's disease. Clin Mol Hepatol 20: 402-405, 2014.
6. Shi Y, Jiang Z, Yang Z, Zheng P, Wei H, Lin Y, Lv G and Yang Q: Clonorchis sinensis infection and co-infection with the hepatitis B virus are important factors associated with cholangiocarcinoma and hepatocellular carcinoma. Parasitol Res 116: 2645-2649, 2017.
7. Blechacz B: Cholangiocarcinoma: Current knowledge and new developments. Gut Liver 11: 13-26, 2017.
8. Razumilava N and Gores GJ: Cholangiocarcinoma. Lancet 383: 2168-2179, 2014.
9. Zhang H, Yang T, Wu M and Shen F: Intrahepatic cholangiocarcinoma: Epidemiology, risk factors, diagnosis and surgical management. Cancer Lett 379: 198-205, 2016.
10. Ng K, Meyerhardt JA, Chan AT, Sato K, Chan JA, Niedzwiecki D, Saltz LB, Mayer RJ, Benson AB III, Schaefer PL, et al.: Aspirin and COX-2 inhibitor use in patients with stage III colon cancer. J Natl Cancer Inst 107: 345, 2014.
11. Rasmussen TI, Flihavan EM, Sharp L, Bennett K and Visvanathan K: Recent prediagnostic aspirin use, lymph node involvement, and 5-year mortality in women with stage I-III breast cancer: A nationwide population-based cohort study. Cancer Res 74: 4065-4077, 2014.
12. Simon TG, Ma Y, Ludvigsson JF, Chong DQ, Giovannucci EL, Fuchs CS, Meyerhardt JA, Corey KE, Chuong RT, Zhang X and Chan AT: Association between aspirin use and risk of hepatocellular carcinoma. JAMA Oncol 4: 1683-1690, 2018.
13. Choi J, Ghoz HM, Peerapathit T, Baichoo E, Addissie BD, Harmsen WS, Therneau TM, Olson JE, Chaiterakaj R and Roberts LR: Aspirin use and the risk of cholangiocarcinoma. Hepatology 64: 785-796, 2016.
14. Guenzle J, Garrelfs N, Goedeld JM and Weerbroek B: Cyclooxygenase (COX) inhibition by acetylsalicylic acid (ASA) enhances antitumor effects of nitric oxide in glioblastoma cell lines. Mol Neurobiol 56: 6946-6955, 2019.
15. Henry WS, Laszewski T, Tsang T, Becfa F, Beck AH, McAllister SS and Toker A: Aspirin suppresses growth in PI3K-mutant breast cancer by activating AMPK and inhibiting mTORC1 signaling. Cancer Res 77: 790-801, 2017.
16. Liu YX, Peng JY, Sun MM, Liu BW, Yang G, Bu YN, Zhao M, Wang TJ, Zhang WY, Yuan HF and Zhang XD: Aspirin inhibits the proliferation of hepatoma cells through controlling GLUT1-mediated glucose metabolism. Acta Pharmacol Sin 40: 122-132, 2019.
17. Huang Z, Fang W, Liu W, Wang L, Liu B, Liu S and Liu S: Aspirin induces Beclin-1-dependent autophagy of human hepatocellular carcinoma cell. Eur J Pharmacol 823: 58-64, 2018.
18. Raza H, John A and Benedict S: Acetylsalicylic acid-induced oxidative stress, cell cycle arrest, apoptosis and mitochondrial dysfunction in human hepatoma HepG2 cells. Eur J Pharmacol 668: 15-24, 2011.
19. Pavlovic N, Rani B, Gerwins P and Heindryckx F: Platelets as key factors in hepatocellular carcinoma. Cancers (Basel) 11: 1022, 2019.
20. Malehmir M, Pfister D, Gallage S, Szydlowska M, Inverso D, Kotsiliti E, Leone V, Peiseler M, Surewaard BJJ, Rath D, et al.: Platelet GPIbα is a mediator and potential interventional target for NASH and subsequent liver cancer. Nat Med 25: 641-655, 2019.
21. Livak KJ and Schmittinger TD: Analysis of relative gene expression using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
22. Kancheva M, Sato Y, Furumichi M, Morishima K and Tanabe M: New approach for understanding genome variations in KEGG. Nucleic Acids Res 47: D950-D959, 2019.
23. Vlachos IS, Zagkanas K, Paraskevopoulos MD, Georgakis G, Karakoumi D, Vergoulis T, Dalamagas T and Hatzigeorgiou AG: DIANA-miRPath v3.0: Deciphering microRNA function with experimental support. Nucleic Acids Res 43: W460-W466, 2015.
24. Cho S, Jang I, Jun Y, Yoon S, Ko M, Kwon Y, Chi O, Chang H, Ryu D, Lee B, et al.: MiRGAitor v3.0: A microRNA portal for deep sequencing, expression profiling and mRNA targeting. Nucleic Acids Res 41 (Database issue): D252-D257, 2013.
25. Huang HY, Lin YC, Li J, Huang KY, Shrestha S, Hong HC, Tang Y, Chen YG, Jin CN, Yu Y, et al.: miRTarBase 2020: Updates to the experimentally validated microRNA-target interaction database. Nucleic Acids Res 48: D148-D154, 2020.
26. Pietenpol JA and Stewart ZA: Cell cycle checkpoint signaling: Cell cycle arrest versus apoptosis. Toxicology 181-182: 475-481, 2002.
27. Wang P, Chi X, Du Q, Luo J, Cui X, Dong K, Bing Y, Heres C and Planer F: miR-383 promotes cholangiocarcinoma cell proliferation, migration, and invasion through targeting IRF1. J Cell Biochem 119: 9720-9729, 2018.
28. Mansini AP, Lorenzo Pisarello MJ, Thelen KM, Cruz-Reyes M, Peixoto E, Jin S, Howard BN, Trussoni CE, Gajdos GB, Karagkouni D, Vergoulis T, Dalamagas T and Hatzigeorgiou AG: Updates to the experimentally validated microRNA-target interaction database. Nucleic Acids Res 48: D148-D154, 2020.
N‑myc downstream‑regulated gene 2 inhibits β: The Cyclins and cyclin‑dependent kinases: 43. 40. 39. 37. 35. 32. 30. 29. 210 inhibition increases menin expression and decreases cholangio Ehrlich L, Hall C, Venter J, Dostal D, Bernuzzi F, Invernizzi P, targeting S100A7. Oncol Lett 15: 386-392, 2018.

Fan F, Lu J, Wu J, Wang Y, Li W, Shao L and Geng Z: MicroRNA-551b-3p inhibits tumour growth of human cholangiocarcinoma by targeting Cyclin D1. J Cell Mol Med 23: 4945-4954, 2019.

Zhang W, Wang Y, Li W, Shi L and Geng Z: MicroRNA-551b-3p inhibits tumour growth of human cholangiocarcinoma by targeting Cyclin D1. J Cell Mol Med 23: 4945-4954, 2019.

Rizvi S and Gores GJ: Pathogenesis, diagnosis, and management of cholangiocarcinoma. Gastroenterology 145: 1215-1229, 2013.

Petrock JL, Sahasrabuddhe VV, Chan AT, Alavanja MC, Beame-Freeman LE, Buring JE, Chen J, Chong DQ, Freedman ND, Fuchs CS, et al: NSAIAD use and risk of hepatocellular carcinoma and intrahepatic cholangiocarcinoma: The liver cancer pooling project. Cancer Prev Res (Phila) 8: 1156-1162, 2015.

Dovizio M, Bruno A, Taconnelli S and Patrignani P: Mode of action of aspirin as a chemopreventive agent. Recent Results Cancer Res 191: 39-65, 2013.

Masaki T, Shiratori Y, Rengofo W, Igarashi K, Yamagata M, Kurokohchi K, Uchida N, Miyauchi Y, Yoshiji H, Watanabe S, et al: Cyclins and cyclin-dependent kinases: Comparative study of hepatocellular carcinoma versus cirrhosis. Hepatology 37: 534-543, 2003.

Zhang X, Feng H, Li Z, Guo J and Li M: Aspirin is involved in the cell cycle arrest, apoptosis, cell migration, and invasion of oral squamous cell carcinoma. Int J Mol Sci 19: 2029, 2018.

Pozzoli G, Marei HE, Alithani A, Boninsegna A, Casalbore P, Marlier LNIL, Lanzilli G, Zonfrillo M, Petrucci G, Rocca B, et al: Aspirin inhibits cancer stem cells properties and growth of glioblastoma multiforme through Rb1 pathway modulation. J Cell Physiol: Jan 30, 2019 (Epub ahead of print).

Hossain MA, Kim DH, Jang YJ, Kang YJ, Yoon JH, Moon JQ, Chung HY, Kim GY, Choi YH, Coppole BL and Kim ND: Aspirin induces apoptosis in vitro and inhibits tumour growth of human hepatocellular carcinoma cells in a nude mouse xenograft model. Int J Oncol 40: 1298-1304, 2012.

Choi BH, Chakraborty G, Baek K and Yoon HS: Aspirin-induced Bel-2 translocation and its phosphorylation in the nucleus trigger apoptosis in breast cancer cells. Exp Mol Med 45: e47, 2013.

Lu TX and Rothenberg ME: MicroRNA. J Allergy Clin Immunol 141: 1202-1207, 2018.

Paliouras AR, Montevedere T and Garofalo M: Oncogene-induced regulation of microRNA expression: Implications for cancer initiation, progression and therapy. Cancer Lett 421: 152-160, 2018.

Lu G and Zhang Y: MicroRNA-340-5p suppresses non-small cell lung cancer cell growth and metastasis by targeting ZNF503. Cell Mol Biol Lett 24: 34, 2019.

Rongxin S, Pengfei L, Li S, Xiaochen J and Yihe H: MicroRNA-340-5p suppresses osteosarcoma development by down-regulating the Wnt/β-catenin signaling pathway via targeting the STAT3 gene. Eur Rev Med Pharmacol Sci 23: 982-991, 2019.

Yue W, Zheng X, Lin Y, Yang CS, Xu Q, Carpizo D, Huang H, DiPaola RS and Tan XL: Metformin combined with aspirin significantly inhibit pancreatic cancer cell growth in vitro and in vivo by suppressing anti-apoptotic proteins Mcl-1 and Bel-2. Oncotarget 6: 21208-21224, 2015.