

Apigenin inhibits renal cell carcinoma cell proliferation through G2/M phase cell cycle arrest

YUHANG BAO¹, XIUXIAN WU¹, XINGHUA JIN¹, AKIHIRO KANEMATSU¹,
MICHIO NOJIMA¹, YOSHIYUKI KAKEHI² and SHINGO YAMAMOTO¹

¹Department of Urology, Hyogo College of Medicine, Hyogo 663-8501; ²Department of Urology, Kagawa University Faculty of Medicine, Kagawa 761-0793, Japan

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Abstract. Apigenin is a flavonoid widely presented in fruits and vegetables, and is known to possess anti-inflammatory, antioxidant, and anticancer properties. The present study was designed to investigate the effects of apigenin on renal cell carcinoma (RCC) cells. These effects on cell growth were evaluated using a cell counting kit, while cell cycle distribution was investigated by flow cytometry following propidium iodide DNA staining. The human RCC cell lines, Caki-1, ACHN, and NC65, were each treated with 1-100 μ M apigenin for 24 h, which resulted in concentration-dependent cell growth inhibition, with the effects confirmed by trypan blue staining. Furthermore, even when the apigenin treatment period was shortened to 3 h, the same cytostatic effect on RCC cells was noted. Similarly, a concentration-dependent cell growth inhibitory effect was also observed in primary RCC cells, as apigenin induced G2/M phase cell cycle arrest and reduced the expression levels of cyclin A, B1, D3, and E in RCC cells in both dose- and time-dependent manners. These findings suggest the possibility of the use of apigenin as a novel therapeutic strategy for treatment of RCC due to its anticancer activity and ability to function as a cell cycle modulating agent.

Introduction

Renal cell carcinoma (RCC), the most common malignancy found in adult kidneys, is the tenth leading cause of cancer-related mortality in Western countries, and is known to be resistant to chemotherapy and radiation therapy (1,2). Although agents used for molecular-targeted therapy, including sunitinib, axitinib, temsirolimus, and pazopanib, have significantly prolonged the survival of patients with advanced

RCC, responses induced by these drugs are transient (3-5). Additionally, the recent advent of immunotherapy with the use of an immune checkpoint inhibitor (ICI), such as nivolumab, pembrolizumab, ipilimumab, and avelumab, has resulted in the possibility of obtaining significant antitumor activity with prolonged and durable responses in metastatic RCC patients, although reported complete response rates induced by these drugs range only from 4 to 10% (6-8). Furthermore, a related concern is the occurrence of immune-related adverse events (irAEs), which can have effects on nearly all organs with varying frequency and severity, such as hypophysitis, thyroiditis, adrenalitis, hepatitis, interstitial pneumonitis, colitis, and interstitial nephritis (9). Therefore, development of novel and effective therapeutic strategies for metastatic RCC is an urgent need.

Apigenin, a natural flavonoid, is widely distributed in a variety of fruits and vegetables, and this particular natural compound has been shown to have a low level of toxicity as well as potential antioxidant, anti-inflammatory, and anticancer properties (10-14). It has also been reported that apigenin inhibited tumor proliferation *in vitro* and *in vivo* in examinations of several different types of human cancer cell lines, including those associated with lung (15), prostate (16,17), leukemia (18), breast (19), pancreatic (20), and oral cancer (21). This flavonoid is considered to be a potentially effective anticancer agent because it exhibits selective induction of cell cycle arrest and apoptosis in tumor cells without affecting normal cells (22,23).

The present study was conducted to investigate the anticancer activity of apigenin toward RCC cells in experiments conducted with three human RCC cell lines as well as primary RCC cells obtained from five patients. In addition, molecular mechanisms possibly involved in the anticancer activity of apigenin toward RCC cells were explored.

Materials and methods

Reagents. Apigenin was purchased from Sigma-Aldrich/Merck KGaA and a Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Laboratories. Apigenin was dissolved in dimethylsulfoxide (DMSO) and subsequently diluted in culture medium. The DMSO concentration did not exceed 0.1% during treatment.

Correspondence to: Dr Xiuxian Wu, Department of Urology, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan
E-mail: wuxian@hyo-med.ac.jp

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RCC cell lines and primary RCC cells. The human RCC cell lines ACHN, Caki-1, and NC65 (ATCC) were used. Primary RCC cells were separated from surgical specimens obtained from five patients with untreated RCC, as previously described (24). Pathologic stage and grade were consistent with the 2004 WHO criteria (<https://www.patologi.com/WHO%20kidney%20testis.pdf>), as follows: T2N0M0 grade 1 in patient 1 (70 years, male); T3bN0M0 grade 2 in patient 2 (68 years, female); T2N0M0 grade 1 in patient 3 (70 years, male); T3bN0M1b grade 2 in patient 4 (76 years, female); and T2N0M0 grade 2 in patient 5 (63 years, male) at Hyogo College of Medicine Hospital between March 1997 and February 1998. All cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin and streptomycin, and then maintained at 37°C in 5% CO₂.

Ethical approval for the use of human tissue was granted by the Hyogo College of Medicine (Hyogo, Japan). All patients provided individual written informed consent for the use of their sampled tissues.

Cytotoxicity assays. Cytotoxicity was determined based on colorimetric assay findings of cell viability obtained with a CCK-8 Kit (Dojindo Laboratories). Briefly, a 100- μ l suspension of 0.5×10^4 cells was seeded into a 96-well flat bottom microtiter plate. After incubation for 24 h, a drug solution or medium alone (control) was added to the plates in triplicate, and then each plate was incubated for an additional 24 h. Subsequently, 10 μ l of CCK-8 solution was added for 3 h. Absorbance (A) in each well was measured using a SPECTRAmax PLUS384 (Molecular Devices, LLC) at 450 nm as the reference, and cell viability was determined based on the percentage of control cells using the following formula: [Percent cell viability = (A of treated wells/A of control wells) x 100] (25).

Cell viability and morphologic changes were evaluated using trypan blue dye exclusion test and phase-contrast microscopy findings, respectively. Cells were seeded into a 6-well plate at 1.5×10^5 cells per well and cultured for 24 h, and then treated in duplicate with apigenin for 24 h. After treatment, the cells were harvested and viability was assessed after 0.5% trypan blue dye (Sigma-Aldrich/Merck KGaA) staining for 1 min at room temperature, and then counted using a hemocytometer under a phase contrast microscope as previously reported (26). Cell death was observed and photographs of adherent cells were obtained using phase-contrast microscopy after removal of medium containing floating cells.

Cell cycle analysis. Cells were treated with apigenin for 24 h, then harvested, washed twice with cold assay buffer, and processed for cell cycle analysis. Briefly, the cells were fixed in cell cycle phase determination fixative at room temperature and stored at -20°C overnight for later analysis according to the protocol of the manufacturer (cell cycle phase determination kit, no. 10009349, Cayman Chemical). Fixed cells were centrifuged at 1,500 rpm and washed with cold PBS twice. Next, RNase A (20 μ g/ml final concentration) and propidium iodide (PI) staining solution (20 μ g/ml final concentration) were added, and the cells were incubated for 30 min at room temperature in the dark. Analysis was performed using a LSRFortessa™ X-20 instrument (BD Biosciences) equipped with BD FACSDiva software. Furthermore, FlowJo v10.7.1 (FlowJo LLC) trial cell

cycle analysis software was used to determine the percentage of cells in each of the different cell cycle phases.

Western blot analysis. Cells were plated in 10-cm plates for 24 h and then treated with apigenin for 6–24 h in a cell culture incubator at 37°C. Following the indicated treatment, cells were lysed for 30 min on ice in lysis buffer with a protease inhibitor cocktail, and then protein concentrations were determined using a Bradford Assay Kit (Bio-Rad Laboratories). Next, 20 μ g of protein was separated using 10% SDS-PAGE and transferred to a PVDF membrane. After blocking nonspecific binding sites for 2 h at room temperature with 5% skim milk in TBS with 0.1% Tween-20, the membranes were incubated overnight at 4°C with the following primary antibodies: cyclin-dependent kinase 1 (CDK1; bs-0542R, Bioss Inc.), cyclin A (sc-271682), cyclin B1 (sc-245), cyclin D1 (sc-6281), cyclin D3 (sc-6283), and cyclin E (sc-247) from Santa Cruz Biotechnology, Inc. at 1:200 dilution, and β -actin mouse polyclonal [E4D9Z, Cell Signaling Technology, Inc. (CST)] at 1:2,000 dilution. The membranes were washed three times by TBST buffer for 30 min at room temperature and then incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody (code. 330, MBL) at 1:2,000 dilution and HRP-conjugated goat anti-rabbit IgG secondary antibody (sc-2004, Santa Cruz Biotechnology, Inc.) at 1:1,000 dilution for 1 h at room temperature. Finally, the membranes were washed three times with TBST buffer for 30 min at room temperature and signals were detected using chemiluminescence ECL kit (GE Healthcare) with an ImageQuant LAS 4010 system (GE Healthcare).

Statistical analysis. All determinations were conducted at least three times, and the results are expressed as the mean \pm SD of three experiments. All analyses were performed using Graphpad Prism V8 for Mac (GraphPad Software, Inc.). A two-tailed value of $P < 0.05$ was considered to indicate statistical significance. Differences between treatment groups and controls were analyzed by one-way ANOVA analysis of variance, followed by Dunnett's multiple comparison test.

Results

Apigenin inhibits cell proliferation. First, the effects of different concentrations of apigenin on cell viability were examined using Caki-1, a human RCC cell line. For cells treated for 24 h, apigenin inhibited proliferation in a dose-dependent manner with a 50% inhibition concentration (IC₅₀) value of 27.02 μ M. Similar antiproliferative effects were also noted with the RCC cell lines ACHN and NC65 (Fig. 1A), which showed IC₅₀ values of 50.40 and 23.34 μ M, respectively. Furthermore, apigenin inhibited proliferation of Caki-1 cells in a time-dependent manner (Fig. 1B). This antiproliferative activity of apigenin was further confirmed by findings obtained with a trypan blue dye-exclusion test (Fig. 1C). Furthermore, a marked decrease in cell numbers, cell swelling, and destruction of cells were also observed using phase-contrast microscopy when the cells were treated with apigenin (Fig. 1D).

To further examine the antiproliferative effect of apigenin on RCC cells, we examined primary RCC cells obtained from five patients. In all patient samples, a marked

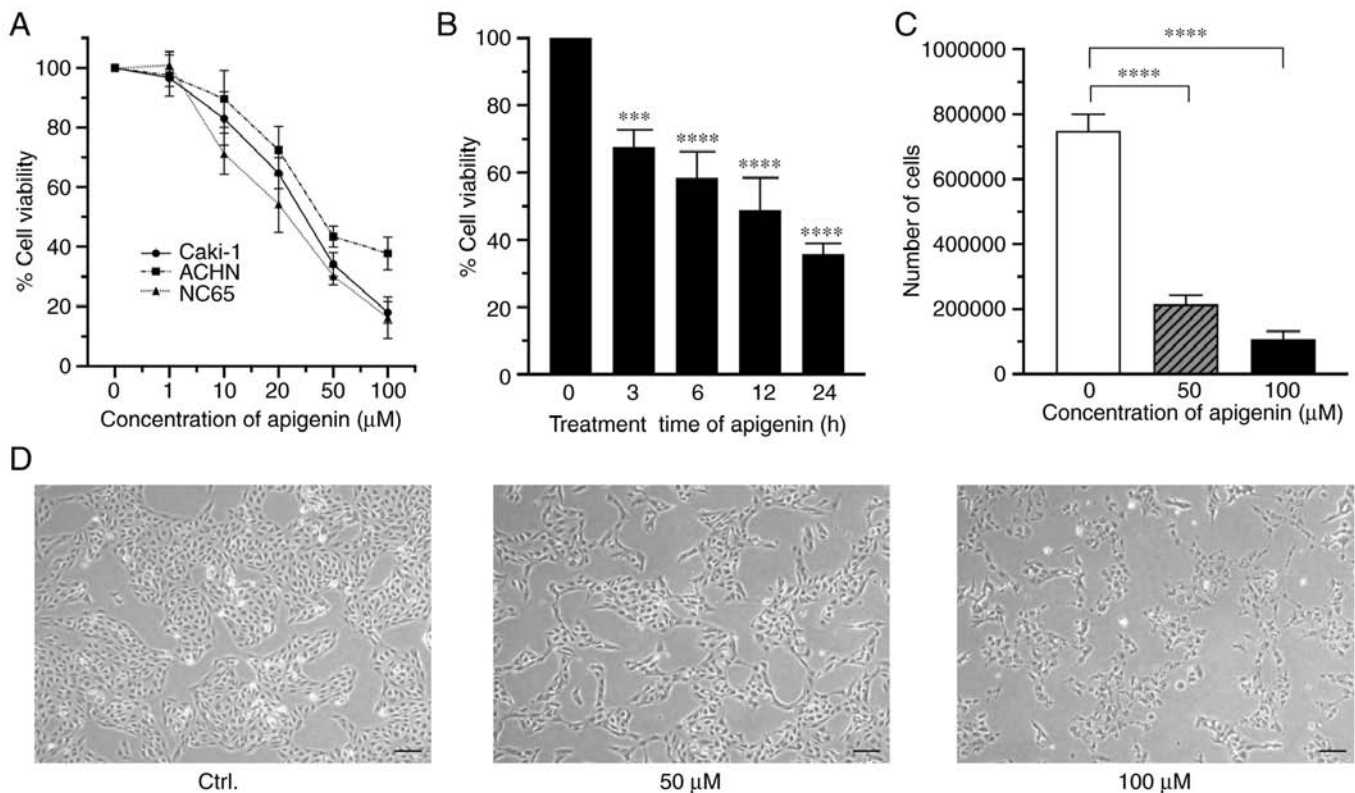


Figure 1. Apigenin inhibits the cell proliferation of human RCC cell lines. (A) Caki-1, ACHN, and NC65 cells were treated with 1-100 μ M apigenin for 24 h, and then (B) Caki-1 cells were treated with 50 μ M apigenin for 3-24 h. Cell viability was determined using a CCK-8 kit. Cells were treated with apigenin (50 or 100 μ M) for 24 h. (C) Cell number was determined using a trypan blue dye exclusion test and (D) cellular morphology was monitored by optical microscopy (x40 magnification), scale bar, 50 μ m. Values are shown as the mean \pm SD of three individual experiments. ***P<0.001, ****P<0.0001 vs. untreated control.

dose-dependent antiproliferative effect was achieved, regardless of the varying sensitivity of the RCC cells (Fig. 2). The IC_{50} values for apigenin with cells from those five cases (patient 1-5) were 73.02, 43.74, 35.63, 26.80, and 53.51 μ M, respectively.

Taken together, these findings clearly demonstrated that treatment of human RCC lines as well as primary RCC cells with apigenin provides an antiproliferative effect.

Apigenin induces G_2/M phase cell cycle arrest. In order to better understand the mechanism of cell proliferation inhibition, cell cycle distribution in different phases of the cell cycle was analyzed following apigenin treatment. There were marked changes in the cell cycle shown by Caki-1 cells treated with apigenin including an increase in percentage of cells in the G_2/M phase, along with a concomitant decrease in those in the G_0/G_1 and S phases as compared with untreated cells (Fig. 3A). Following treatment with apigenin at 20, 30, and 50 μ M, the percentage of Caki-1 cells in the G_2/M phase was 22.4, 34.8, and 48.9%, respectively, while only 24% of the control cells were found to be in the G_2/M phase (Fig. 3B).

Apigenin modulates expression of cyclin A, B1, D3, and E. To further assess the molecular mechanisms related to inhibition of cell proliferation, the effects of apigenin on expression levels of cyclin A, B1, D1, D3, and E, as well as CDK1 in RCC cells were evaluated. Apigenin significantly reduced cyclin A, B1, D3 and E expression levels in Caki-1 cells (Fig. 4A-D), whereas there was no effect on cyclin D1 or CDK1 expression

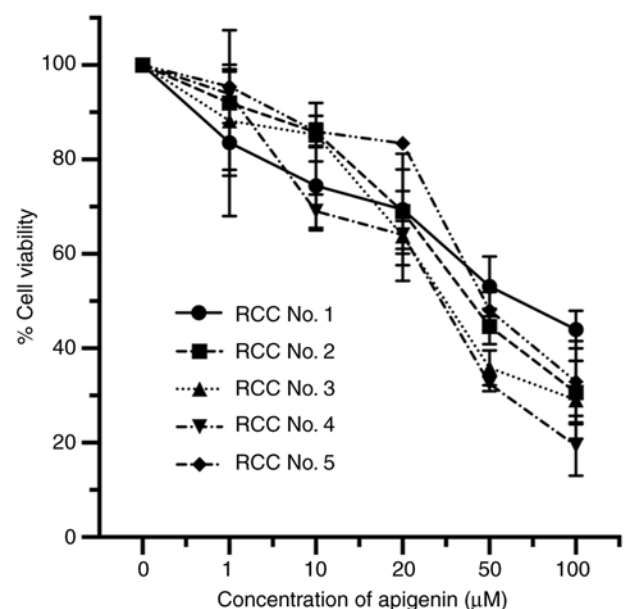


Figure 2. Antiproliferative effects of apigenin on primary RCC cells. Primary RCC cells obtained from five patients were treated with apigenin (1-100 μ M) for 24 h. Cell viability was determined using a CCK-8 kit. Values are shown as the mean \pm SD of three individual experiments.

noted. Additionally, downregulation of cyclin A, B1, D3, and E expression was also observed in the primary RCC cells (Fig. 4E and F).

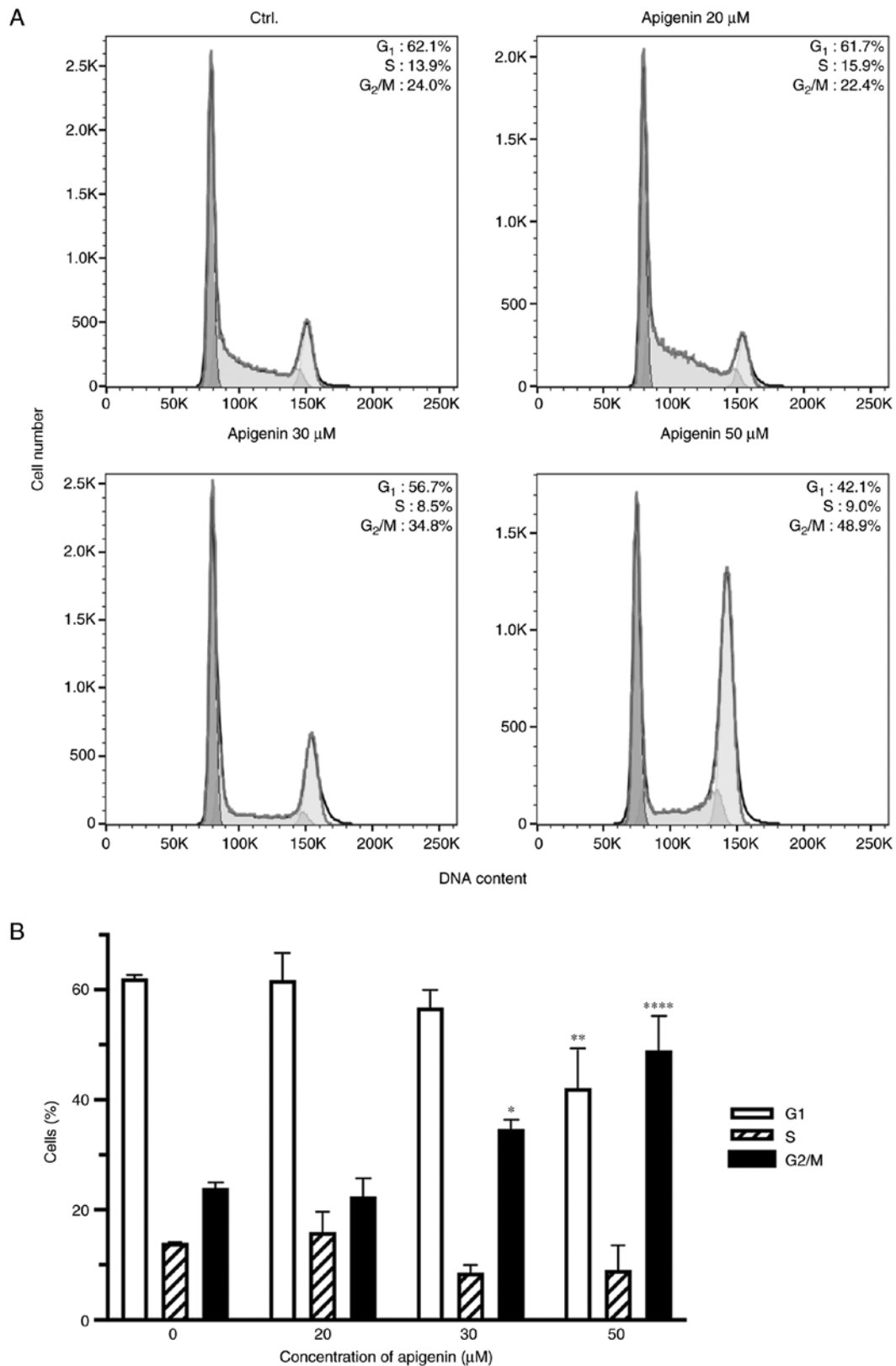


Figure 3. Effects of apigenin on cell cycle distribution in RCC cells. Caki-1 cells were treated with apigenin (20–50 μ M) for 24 h. Cell cycle distribution was determined by flow cytometry using an LSRFortessa X-20 instrument. (A) Representative findings from one of three different experiments are shown. (B) Values are shown as the mean \pm SD of three individual experiments. * P <0.05, ** P <0.01, **** P <0.0001 vs. untreated control.

Discussion

Renal cell carcinoma (RCC) malignancy is highly resistant to conventional chemotherapy and radiation therapy. Durable

responses to targeted agents are rare, and most patients with metastatic RCC eventually progress and die from the disease, even though several molecular-targeted drugs administered to slow RCC growth are currently being used, with some instances

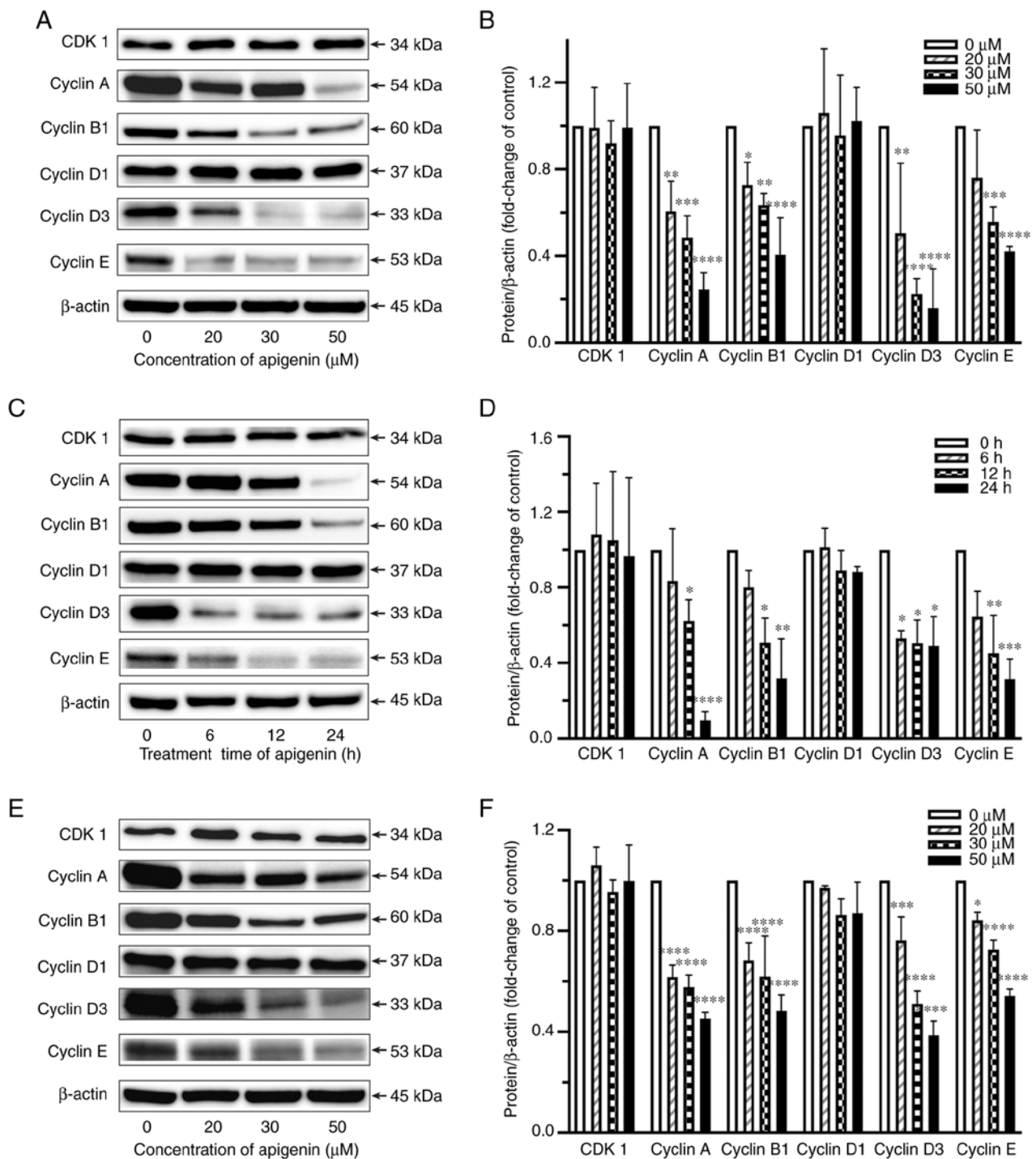


Figure 4. Effects of apigenin on expression levels of CDK1 and cyclin A, B1, D1, D3, and E in RCC cells. Caki-1 cells were treated with apigenin at 20-50 μ M for 24 h (A and B) or at 30 μ M for 6-24 h. (C and D) Primary RCC cells (RCC No. 1) were treated with apigenin at 20-50 μ M for 24 h. (E and F) Expression levels of CDK1 and cyclin A1, B1, D1, D3, and E were assessed by western blotting. β -actin was used as the loading control. Representative findings from one of three individual experiments are shown. Densitometric analysis of blots of protein were normalized to the corresponding β -actin levels. Values are shown as the mean \pm SD of three individual experiments. * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001 vs. untreated control.

of success reported (27,28). Recently, immune checkpoint inhibitors (ICIs) have been shown to have significant anti-tumor activity, with prolonged and durable responses noted in metastatic RCC cases, although the complete response rate induced by these drugs is less than 10% (6,7). Furthermore, a matter of concern is the wide range of immune-related adverse events (irAEs) that can affect nearly all organs with varying frequency and severity in patients receiving ICIs (9). Thus,

development of novel and effective therapeutic strategies for metastatic RCC is an urgent need.

The present results demonstrated that apigenin, a flavonoid widely present in fruits and vegetables, has antiproliferative effects toward RCC cells, which were observed in experiments with not only established human RCC cell lines but also human primary RCC cells. The antiproliferative activity of apigenin was demonstrated by induction of G2/M phase cell

cycle arrest. From a clinical perspective, our findings suggest that apigenin is a promising agent.

The anticancer action of apigenin is dependent on various mechanisms that can vary according to cell type and involve apoptosis, modulation of the cell cycle, and alteration of kinase pathways (14). In the present study, analysis of cell cycle distribution revealed a marked increase in the percentage of RCC cells in the G2/M phase following apigenin treatment. These results support those of previous studies showing that apigenin inhibits the cell cycle of various types of human cancer cells in the G2/M phase (20,29-32).

Cell cycle checkpoints at G2/M as well as G1/S are critical for maintaining DNA integrity, and also regulating the passage of cells through the cell cycle. It is well known that loss of these checkpoints is involved in the process of transformation into cancer cells and disease progression. A protein kinase complex consisting of CDK1, a catalytic subunit, and cyclin B has a central rate-limiting function in the transition from G2 to M phase (33,34). This CDK1/cyclin B complex responds to DNA damage and causes a delay in cell cycle progression, which allows for DNA repair before the cells enter mitosis. Several investigators have also shown that the combination of CDK1 with cyclin A and B is critical for G2/M phase transition (35,36). On the other hand, it has been reported that keratinocyte cells do not show modulation of the CDK1 level after apigenin treatment (21), thus indicating that apigenin induces G2/M arrest through a variety of mechanisms in different cells, and that cell growth deregulation in cancer cells may be dependent on the downregulation of cyclin E expression (21,37). In the present experiments, apigenin remarkably reduced the expression of cyclin A, B1, D3 and E in RCC cells, whereas it had no effect on expression of CDK1 or cyclin D1. Thus, downregulation of cyclin B1, as well as cyclin A, D3 and E by apigenin may have been the main cause of G2/M phase arrest observed in the RCC cells. This decrease in quantity of cyclins observed as a result of apigenin treatment is consistent with arrest during the G2/M phase, because these proteins are not expressed in resting cells (38).

Based on findings demonstrating a relatively selective growth inhibitory effect toward cancer cells as compared with normal cells, apigenin is considered to be an attractive candidate for cancer treatment (22). Shukla *et al* reported significantly delayed development of prostate cancer in mice following apigenin administration as well as delayed occurrence of death from prostate cancer (17). It was also demonstrated that apigenin inhibits melanoma lung metastasis by impairing the interaction of tumor cells with the endothelium (39). Recently, Meng *et al* reported reduced tumor growth and volume *in vivo* in an ACHN cell xenograft mouse model administered apigenin treatment (33). In addition, no severe side effects of apigenin administration have been observed in studies of mice that used therapeutic doses (17,33,38,40). The present results demonstrated that apigenin has antiproliferative effects, not only in human RCC cell lines but also human primary RCC cells. Notably, even when the treatment time was shortened from 24 to 3 h, the same cytostatic effect was shown in the RCC cells. These results suggest that apigenin may be useful for development as an effective therapeutic agent for advanced RCC. Additionally, the present study also observed

that the IC₅₀ values for apigenin in primary RCC cells from those five cases (patient 1-5) were the strong different, thus may indicate different sensitivities of apigenin in different patients. Furthermore, the present findings showing a cytostatic effect on RCC cells treated with apigenin for a short period of time may provide a foundation for optimizing administration in clinical applications. Further study will be needed to confirm its antitumor activity using primary RCC cell xenograft mouse models, as the limitations of the current study include lack of an *in vivo* mouse model.

In conclusion, apigenin was shown to have an antiproliferative effect and induce G2/M phase arrest in RCC cells, possibly through direct downregulation of cyclin A, B1, D3 and E. Together, the present findings suggest treatment of RCC with apigenin as a promising potential clinical application.

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Availability of data and materials

All data generated or analyzed during this study are included in the current published article.

Authors' contributions

YB performed the cell proliferation and western blot assays, and statistical analysis of the data obtained in all of the experiments. XJ and AK performed the trypan blue staining and cell cycle analysis. MN and YK analyzed and interpreted the cell proliferation assay data. SY and XW planned, analyzed, and interpreted all of the experiments and validity of the data, and drafted the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work and data are appropriately investigated and resolved.

Ethics approval and consent to participate

Ethical approval for the use of human tissue was granted by the Hyogo College of Medicine (approval no. 202104-07, Hyogo, Japan). All patients provided individual written informed consent for the use of their sampled tissues.

Patient consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interests.

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