Apigenin inhibits growth and induces apoptosis in human cholangiocarcinoma cells

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Received April 26, 2016; Accepted February 1, 2017

DOI: 10.3892/ol.2017.6705

Abstract. A promising nutraceutical, apigenin, was recently revealed to exhibit biological activity in inhibiting several types of cancer. The effects of apigenin on the growth inhibition and apoptosis of the cholangiocarcinoma HuCCA-1 cell line were investigated. Protein alterations subsequent to apigenin treatment were studied using a proteomic approach. The values of 20, 50 and 90% inhibition of cell growth (IC20, IC50 and IC90) were determined by MTT cell viability assay. Apoptotic cell death was detected using two different methods, a flow cytometric analysis (Muse Cell Analyzer) and DNA fragmentation assay. A number of conditions including attached and detached cells were selected to perform two-dimensional gel electrophoresis (2-DE) to study the alterations in the expression levels of treated and untreated proteins and identified by liquid chromatography (LC)/tandem mass spectrometry (MS/MS). The IC20, IC50 and IC90 values of apigenin after 48 h treatment in HuCCA-1 cells were 25, 75 and 200 µM, respectively, indicating the cytotoxicity of this compound. Apigenin induced cell death in HuCCA-1 cells via apoptosis as detected by flow cytometric analysis and exhibited, as confirmed with DNA fragmentation, characteristics of apoptotic cells. A total of 67 proteins with altered expression were identified from the 2-DE analysis and LC/MS/MS. The cleavage of proteins involved in cytoskeletal, cytokeratin 8, 18 and 19, and high expression of S100-A6 and S100-A11 suggested that apoptosis was induced by apigenin via the caspase-dependent pathway. Notably, two proteins, heterogeneous nuclear ribonucleoprotein H and A2/B1, disappeared completely subsequent to treatment, suggesting the role of apigenin in inducing cell death. The present study indicated that apigenin demonstrates an induction of growth inhibition and apoptosis in cholangiocarcinoma cells and the apoptosis pathway was confirmed by proteomic analysis.

Introduction

A nutraceutical is a food or a part of a food that provides medicinal and health benefits (1). Several nutraceuticals originate from natural sources. Understanding the action of these active nutritional compounds and their mechanisms associated with their health benefits is of interest. Apigenin, 4',5,7-trihydroxyflavone, is a promising food-based flavonoid compound present abundantly in common fruits and vegetables including oranges, parsley, celery, spearmint, tea, wheat sprouts, perennial chamomile, onions and a number of seasonings (2). It has been demonstrated to display a variety of biological activities, including anti-inflammatory, anti-oxidant, anticarcinogenic, chemoprevention and tumor growth inhibition (3,4).

The higher incidence of cholangiocarcinoma (CCA), a malignant tumor derived from intrahepatic or extrahepatic biliary tracts, occurs in Southeast Asian countries such as Thailand (5). Congenital liver malformations, primary sclerosing cholangitis and infection with the parasitic liver flukes Opisthorchis viverrini are risk factors for cholangiocarcinoma (6). The risk of cholangiocarcinoma increases in patients with chronic liver disease with either form of viral hepatitis, B or C (7,8), alcoholic liver disease or cirrhosis from a number of causes (9,10). Our group has established the proteomic map of a Thai human cholangiocarcinoma HuCCA-1 cell line and compared it to Thai human hepatocellular carcinoma HCC-S102 cell line and hepatoblastoma HepG2 cell line by studying their soluble proteins (11) and membrane proteins (12).

Apoptosis, a process of programmed cell death in multicellular organisms, is one of the main types of cell death pathway and involves a series of biochemical events, which lead to cell morphology and mortality (13). When the apoptotic process occurs, the cell body and fragments are safely disposed. This serves a critical role in the multiple steps of tumorigenesis. The specific proteolytic activities of caspasas, cysteinyl-aspartate proteases, are recognized to be responsible for many of these
morphic alterations (14,15). Several proteins are known to potentially inhibit (16) or promote (17) the onset of apoptosis by a number of means of activation. Several studies have focused on apoptosis-associated proteins in apoptotic cells (18,19).

The use of apigenin as an anticancer agent in vitro for the treatment of various cancer cells including prostate, breast, cervical, lung, tongue oral, leukemia and colorectal cancer has increased (20-22). The evidence of apigenin-induced apoptosis has been demonstrated in a number of cancer cell lines but there is no study on the anticancer action of apigenin on cholangiocarcinoma cell lines.

In the present study, MTT assays were performed to study the cytotoxicity of apigenin on a cholangiocarcinoma cell line, and flow cytometric analysis was employed to determine the induction of apoptosis. The proteomic analysis was also used to study the differential protein expression between apigenin-treated and untreated cells.

Materials and methods

Cell culture. The HuCCA-1 cell line, derived from a bile duct tumor mass, was provided by Professor Stitaya Sirisinha, Faculty of Science, Mahidol University (Bangkok, Thailand) and grown as a monolayer culture in Ham's F12 culture medium (Gibco Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA), containing 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories; GE Healthcare Life Sciences, Logan, UT, USA), 100 U/ml penicillin, 100 mg/ml streptomycin and 125 ng/ml amphotericin B. The cells were maintained at 37°C in a humidified atmosphere with 5% CO2.

Cytotoxicity assay. Cells at 80% confluence were harvested by trypsinization from culture flasks and seeded in 96-well plates at 10^4 cells per 100 µl per well. After 24 h incubation, the cells were treated with apigenin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at various concentrations (1-250 µM) for 24, 48 and 72 h. Each well was then replaced with fresh medium containing 0.5 mg/ml MTT (Sigma-Aldrich; Merck KGaA) and incubated for 2 h. Finally, the medium was removed and 100 µl dimethyl sulfoxide was added to each well. The absorbance was measured at 550 nm with a microplate reader, subtracted with the absorbance at 650 nm. Data were expressed as % cell growth compared with the untreated cells as the control.

Detection of apoptosis. Apoptosis was detected by two different methods, flow cytometric analysis of phosphatidylserine externalization and a DNA fragmentation assay. For the flow cytometric analysis, the HuCCA-1 cells were seeded in 6-well plate at 4×10^5 cells per 2 ml per well. After 24 h incubation, the cells were treated with apigenin at concentrations of 20% inhibition of cell growth (IC50), 25 µM, IC50, 75 µM and IC50, 200 µM, respectively. After 48 h of compound treatment, floating cells in culture media were harvested by trypsinization, then the two cell populations were pooled together and centrifuged at 778 x g for 10 min at 4°C. The supernatant was removed and the cell pellets were resuspended and adjusted to 1×10^6 cells/ml in culture media containing 1% FBS. Equal volumes of the cell suspension and reagent of Muse™ Annexin-V & Dead Cell kit (Merck KGaA) were mixed together in a tube and incubated at room temperature for 20 min, and analysis was performed using Muse™ Cell Analyzer (Merck KGaA) (23). For the DNA fragmentation assay, after 48 h of compound treatment, floating cells in culture media were harvested by centrifugation at 778 x g for 10 min at 4°C, whilst adherent cells were harvested by scraping in cold 1X PBS followed by the centrifugation at 778 x g for 10 min to collect the cells. Subsequent to this step, the cell pellets were subjected to DNA extraction using the QIAamp DNA kit (Qiagen GmbH, Hilden, Germany), the isolated DNA fragments were resolved in 2% agarose gel using electrophoresis and then visualized by staining with ethidium bromide.

Sample preparation and protein extraction. Tissue culture flasks measuring 75 cm² were used for seeding HuCCA-1 cells and the cells were cultured at 37°C, 5% CO2 for 24 h. Apigenin was then added to the cells at final concentration of 200 µM, 90% inhibition, IC50, 48 h. Since apigenin treatment causes cell detachment, the floating cells were collected from the medium by centrifugation at 778 x g for 10 min at 4°C and washed with 0.25 M sucrose-containing protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA). The adherent cells were harvested by trypsinization and washed twice with 0.25 M sucrose, scraped in the same sucrose solution and centrifuged at 778 x g for 10 min at 4°C. The two samples were resuspended in 100 µl lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 2% dithiothreitol (DTT), 2% ampholine pH 3-10 and a protease inhibitor cocktail, sonicated on ice and centrifuged at 13,800 x g for 10 min at 4°C. The supernatants were saved and the concentration of proteins was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Two-dimensional gel electrophoresis. The samples were prepared by leaving them overnight in gel rehydration of nonlinear pH 3-10, 70-mm Immobline DryStrip gels (IPG; GE Healthcare, Chalfont, UK). An Ettan IPGphor system (GE Healthcare) was used for running the first dimension isoelectric focusing at 6,500 Vh. The IPG strips were equilibrated in 1X buffer solution, 0.1 M NH4HCO3, 10 mM DTT and 1 mM acetic acid was used as the staining solution.

Gel scanning and image analysis. The gels were scanned using ImageScanner II (GE Healthcare) and analyzed using ImageMaster 2D platinum software (version 7.0; GE Healthcare) for differential analysis.

In-gel digestion. The triplicate washing step was performed by adding 50 µl 0.1 M NH4HCO3 in 50% acetonitrile (ACN) in excised gel spots and incubating for 20 min at 30°C. The gel pieces were dried completely in SpeedVac (Labconco, Kansas City, MO, USA). The gel pieces were reduced and alkylated in 1X buffer solution, 0.1 M NH4HCO3, 10 mM DTT and 1 mM...
EDTA, and incubated at 60°C for 45 min. The buffer solution was replaced with freshly prepared 100 mM iodoacetamide in 0.1 M NH₄HCO₃ solution. The reaction mixture was incubated in the dark at room temperature for 30 min. The gel pieces were washed three times using 50% ACN in water and were dried completely. The trypsin (Promega Corporation, Madison, WI, USA) was aliquoted (1 μg trypsin/10 μl 1% acetic acid) and stored at -20°C. The digestion buffer, 0.05 M Tris-HCl, 10% ACN, 1 mM CaCl₂, pH 8.5, was prepared. The tryptic digestion was performed by adding 50 μl digestion buffer and 1 μl prepared trypsin into the gel pieces. The reaction mixture was incubated at 37°C overnight. The digestion buffer was removed and saved. The gel pieces were then added to 60 μl 2% freshly prepared trifluoroacetic acid and incubated at 60°C for 30 min for peptide extraction. The saved digestion buffer and the final extract were then pooled and dried by SpeedVac.

**Protein identification by liquid chromatography (LC) tandem mass spectrometry (MS/MS).** The Q-TOF mass spectrometer (Micromass UK Ltd., Manchester, UK) equipped with a Z-spray ion source operating in the nanoelectrospray mode was used. The analysis by LC was carried out using a capillary LC system (Waters Corporation, Milford, MA, USA). The instrument in MS/MS mode was calibrated by Glu-fibrinopeptide. The 75 mm id x150 mm C18 PepMap column (LC Packings, Amsterdam, The Netherlands) was attached to the LC system. Eluents A and B were prepared as follows: Eluent A, 0.1% formic acid in 97% water and 3% ACN and eluent B, 0.1% formic acid in 97% ACN and 3% water. The gradient for peptide separation was 0 min 7% B, 35 min 50% B, 45 min 80% B, 49 min 80% B, 50 min 7% B and 60 min 7% B. ProteinLynx Global SERVER™ (version 2.2; Waters Corporation) screening Swiss-Prot and NCBI (www.ncbi.nlm.nih.gov/) was employed for database search. The MASCOT (http://www.matrixscience.com) search tool available on the Matrix Science site screening and NCBIInr was also used to confirm certain proteins.

**Western blot analysis.** The untreated and apigenin treated HuCCA-1 cells were scraped separately and sonicated in an in-house 1X radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0 and 1 mM EDTA) to extract the proteins. Protein lysates (20 μg) were subsequently loaded in each lane. Proteins separation was performed by 12.5% SDS-PAGE and transferred onto FluoroTrans® polyvinylidene difluoride membranes (Pall Corporation, Port Washington, NY, USA). Subsequent to blocking with 5% nonfat dried milk in TBS-Tween 20 (TBST), 10 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween 20, at room temperature for 1 h, the membranes were washed with TBST and incubated with the following primary antibodies: Mouse monoclonal cytokeratin 7 (CK7; cat. no. MAB3554; dilution, 1:2,000; Merck KGaA); mouse monoclonal cytokeratin 8 (CK8; cat. no. MAB3444; dilution, 1:2,000; Merck KGaA); mouse monoclonal cytokeratin 18 (CK18; cat. no. MAB3236; dilution, 1:2,000; Merck KGaA); mouse monoclonal cytokeratin 19 (CK19; cat. no. MAB3238; dilution, 1:2,000; Merck KGaA); mouse monoclonal S100-A6 (cat. no. ab55680; dilution, 1:250; Abcam, Cambridge, UK); rabbit polyclonal S100-A11 (cat. no. 10237-1-AP; dilution, 1:250; Proteintech, Chicago, IL, USA); rabbit monoclonal S100-P (cat. no. ab133554; dilution, 1:1,000; Abcam); mouse monoclonal heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP A2/B1; cat. no. ab6102; dilution, 1:2,000; Abcam); rabbit polyclonal heterogeneous nuclear ribonucleoprotein H (hnRNP H; cat. no. ab10374; dilution, 1:5,000; Abcam); mouse monoclonal annexin A1 (cat. no. MAB3773; dilution, 1:2,000; Merck KGaA); mouse monoclonal annexin A2 (cat. no. ab54771; dilution, 1:20,000; Abcam); rabbit polyclonal annexin A3 (cat. no. ab33068; dilution, 1:2,000; Abcam); mouse monoclonal peroxiredoxin-1 (cat. no. ab58252; dilution, 1:2,000; Abcam); mouse monoclonal prostatlandin E synthase 3 (PTGES3; cat. no. WH0010728M1; dilution, 1:500; Sigma-Aldrich; Merck KGaA); or rabbit monoclonal GAPDH (cat. no. ab75834; dilution, 1:10,000; Abcam) at 4°C overnight. The membranes were then washed with TBST and incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Dako; Agilent technologies, Inc., Santa Clara, CA, USA) at room temperature for 1 h. Following washing with TBST, membranes were visualized by using an enhanced chemiluminescence Western blotting detection kit (Advansta, Menlo Park, CA, USA) and an ImageQuant LAS 4000 mini (GE Healthcare). A total of three experiments were performed for each antibody.

**Statistical analysis.** The mean values and standard deviations of differential expression between treated and untreated cells were calculated. The significance of differences was analyzed by two-tailed unpaired Student’s t tests, and P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Apigenin inhibited the growth of HuCCA-1 cells.** The MTT assay was performed to investigate the growth inhibition and assess the cytotoxic effect of apigenin. HuCCA-1 cells were treated with various concentrations of apigenin ranging from 1-250 μM at different times. The results in Fig. 1A illustrate the dose- and time-dependent inhibition of cell growth by apigenin. Since cell morphology changes were being observed at 48 h subsequent to treatment as demonstrated in Fig. 1B, the results at this time point were chosen to estimate the value of concentrations that will cause IC₅₀, IC₉₀ and IC₉₀. The values of IC₂₀, IC₅₀ and IC₉₀ of apigenin after 48 h treatment were 25, 75 and 200 μM, respectively. After 48 h incubation, the morphology changes were clearly observed in treated cells at concentrations up to 200 μM compared with the control. The treated cells appeared to lack regular shape with boundaries resembling loosely adhered cells.

**Apigenin induced apoptosis in HuCCA-1 cells.** To examine whether apigenin induced apoptotic cell death in HuCCA-1 cells, two different methods were used to detect apoptosis, flow cytometric analysis of phosphatidylserine (PS) externalization and DNA fragmentation assays. When cells undergo apoptosis, the membrane integrity of the cells is lost, allowing penetration of membrane-impermeant dyes such as 7-aminoactinomycin D (7-AAD) and propidium
Table I. Differentially expressed proteins of HuCCA-1 following apigenin treatment.

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Accession no.</th>
<th>Protein names</th>
<th>Theoretical pI/MW</th>
<th>Function</th>
<th>Fold Change$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Q9Y4L1</td>
<td>Hypoxia up-regulated protein 1</td>
<td>5.21/111.3</td>
<td>Chaperone/stress response</td>
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<td>Spot no.</td>
<td>Accession no.</td>
<td>Protein names</td>
<td>Theoretical pI/MW</td>
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<td>P08727</td>
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<td>Metabolism</td>
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<td>P22626</td>
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<td>8.79/35.9</td>
<td>Metabolism</td>
<td>-7.70±0.92$^c$</td>
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</tbody>
</table>

-, downregulated expression following treatment; +, upregulated expression following treatment; D, appearance of proteins upon treatment; ND, disappearance of proteins upon treatment; pl, isoelectric point; MW, molecular weight. *Fold change of protein expression was calculated from three independent experiments.$^aP<0.05,$ $^bP<0.01,$ $^cP<0.001.$

Figure 1. Effect of apigenin on cell growth. (A) Treatment of HuCCA-1 cells by various concentration of apigenin (1-250 µM) for 24, 48 and 72 h. The number of surviving cell was calculated by MTT assay. The presented data are as mean ± standard deviation of three different experiments. (B) The cells were treated with apigenin at concentrations of 20, 50 and 90% growth inhibition, IC$_{20}$, IC$_{50}$ and IC$_{90}$, respectively. The cells were observed by inverted phase-contrast microscopy (magnification, x100).
Figure 2. Apigenin induces apoptosis in HuCCA-1 cells. (A and B) Flow cytometric analysis of apoptotic cells was performed by using Muse™ Cell Analyzer with Muse™ Annexin-V & Dead Cell kit. The treatment of HuCaC-1 cells was performed with 0.2% dimethyl sulfoxide (control) or different concentrations of apigenin (25–200 µM) for 48 h. Dot plots of flow cytometric results were from three independent experiments. Data are presented as mean ± standard deviation of three different experiments. (C) Detection of apigenin induced DNA fragmentation in HuCCA-1 cells. HuCCA-1 cells were treated with 200 µM apigenin, IC_{90}, for 48 h. Genomic DNA samples were isolated from HuCCA-1 cells with or without apigenin, separation was performed on 2% agarose gels, and visualized using ethidium bromide staining. Lane M, DNA marker; lane 1, DNA from untreated cells; lane 2, DNA from the attached cells subsequent to treatment; lane 3, DNA from the floating cells subsequent to treatment; 7-ADD, 7-amino actinomycin D.

Figure 3. Comparison of 2-DE patterns between untreated and treated HuCCA-1 cells with apigenin. (A and B) Illustrate the 2-DE patterns of untreated and treated cells with 200 µM apigenin. NL pH 3-10 Immobiline DryStrip gels were used. Gels were stained with Coomassie Brilliant Blue R-250. Proteins with different expressions are marked by arrows with numbers. Data presented are the representative of three totally different experiments. 2-DE, two-dimensional gel electrophoresis; NL, non-linear.
iodide. Mode of cell death in apigenin-treated HuCCA-1 cells was analyzed. The cells were treated with increasing concentration from IC$_{20}$, 25 µM, IC$_{50}$, 75 µM and IC$_{90}$, 200 µM of apigenin compared with the untreated control for 48 h. Subsequent to the treatment, the cells that were positive for Annexin-V and 7-AAD staining with stages of early and late apoptosis were detected (Fig. 2A). The percentage of total apoptotic cell populations, early and late apoptosis, increased corresponding to the higher concentration of apigenin. The highest proportion of apoptotic cells was ~41%, when cells were treated with apigenin at a concentration of IC$_{90}$ (Fig. 2B).

Additionally, another key feature of apoptosis, DNA fragmentation with a ladder pattern, was also investigated. The HuCCA-1 cells were treated with IC$_{90}$, 200 µM, apigenin for 48 h, and subsequently analyzed for DNA fragmentation. As demonstrated in Fig. 2C, the ladder pattern of DNA fragmentation was observed in floating cells whilst the attached cells exhibited no visible DNA laddering. Taken together, the results of flow cytometric analysis and DNA fragmentation assay consistently indicated that apigenin induced apoptosis in HuCCA-1 cells.

**Differential expression of proteins in HuCCA-1 when treated with apigenin.** From the DNA fragmentation of apigenin-treated HuCCA-1 cells, apoptosis was clearly demonstrated in the floating cells. Thus, the levels of protein expression in the untreated and apigenin-treated cells floating and adherent cells were compared using proteomic techniques. Only the floating cells demonstrated differential protein expression when compared with the untreated cells by 2-DE. Fig. 3A and B illustrates the 2-DE patterns of the untreated and floating cells treated with 200 µM apigenin, 90% inhibition, IC$_{90}$, for 48 h. A total of sixty-seven proteins were revealed to exhibit differential expression and were identified by comparison to the proteins previously examined in the reference map of HuCCA-1 (11) and using LC/MS/MS as summarized in Table I. These proteins were categorized by their functions as follows: Metabolism, cytoskeletal/mobility, protein synthesis and degradation, signal transduction, chaperone/stress response, protection and detoxification, transport/binding proteins, cell cycle, ion channels and DNA replication/gene regulation. The fold changes of proteins were calculated by ImageMaster program and included in Table I.

The expression levels of twelve proteins were not measured in HuCCA-1 cell lysate subsequent to apigenin treatment. These proteins are involved in metabolism, cytoskeletal/mobility, protein synthesis and degradation, chaperone/stress response, protection and detoxification and transport/binding. These proteins are phosphoenolpyruvate carboxykinase (GTP; Table I, spot no. 2), GAPDH (Table I, spot no. 29), ATPase inhibitor (Table I, spot no. 59) and fructose-bisphosphate aldolase A (Table I, spot no. 62), CK19 (Table I, spot no. 17), stathmin (Table I, spot no. 50), cofilin-1 (Table I, spot no. 52), 26S protease regulatory subunit 10B (Table I, spot no. 41), hnRNP A2/B1, heterogeneous nuclear ribonucleoprotein A2/B1; PTGES3, prostaglandin E synthase 3; hnRNP H, heterogeneous nuclear ribonucleoprotein H.

Additionally, another key feature of apoptosis, DNA fragmentation with a ladder pattern, was also investigated. The HuCCA-1 cells were treated with IC$_{90}$, 200 µM, apigenin for 48 h, and subsequently analyzed for DNA fragmentation. As demonstrated in Fig. 2C, the ladder pattern of DNA fragmentation was observed in floating cells whilst the attached cells exhibited no visible DNA laddering. Taken together, the results of flow cytometric analysis and DNA fragmentation assay consistently indicated that apigenin induced apoptosis in HuCCA-1 cells.
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Proteins involved in protein synthesis and degradation demonstrated low or no expression subsequent to apigenin treatment, including 26S protease regulatory subunit 10B, hnRNP H and hnRNP A2/B1. In particular, the 26S protease regulatory subunit 10B is a protein associated with apoptosis, and disappears in the floating cells. The decreased activity of the 26S proteasome and induction of cell death was observed only in breast cancer cells and not in normal cells, treated with *Murraya koenigii* leaf extract (37). The western blot of the present study also demonstrated that expression of hnRNP H and hnRNP A2/B1 disappeared completely subsequent to apigenin treatment. HnRNP proteins are multifunctional, participating in several cellular processes and composed of at least 20 major, high abundant or core hnRNP proteins including A1, A2/B1, B2, C1 and C2 (38). HnRNP A2/B1 has been demonstrated to be involved in splicing, mRNA stability and mRNA transport during the progress of tumorigenesis (39). Apigenin has been identified to bind specifically to the C-terminal glycine-rich domain of hnRNP A2, which is suggested to prevent hnRNP A2 from forming homodimers, resulting in alternative splicing of a number of human hnRNP A2 targets (40). HnRNP H and hnRNP C1/C2 are also involved in controlling numerous splicing decisions. The lower expression levels of hnRNP proteins upon treatment with apigenin may lead to the elimination of the splicing forms which inhibit cell death and promotion of the normal splice forms in the cells.

Chaperone/stress response proteins including prostaglandin E synthase 3, hypoxia upregulated protein 1 and 60 kDa heat shock protein, were revealed to be downregulated subsequent to treatment with apigenin. However, stress-induced-phosphoprotein 1 was upregulated. Molecular chaperones are involved in protein folding, transport and assembly (41) and function to maintain cell survival. Elevated expression levels of heat shock proteins may promote cancer and may lead to resistance to chemotherapy and hyperthermia (42). It has been suggested that heat shock proteins and their co-chaperones are involved in the regulation of apoptosis by caspase activation (43). Stress-induced-phosphoprotein 1 (STIP1) is an Hsp70/Hsp90-organizing protein, a co-chaperone that regulates the different functions of Hsps. STIP1 has been associated with several types of cancer (44-47). PTGES3 or cytosolic prostaglandin E synthase is a 23 kDa glutathione-requiring enzyme expressed in a wide variety of cells. It is identical to co-chaperone p23 that binds to heat shock protein 90 (Hsp90) (48). PTGES3/p23 inhibited ATPase activity to stabilize the closed conformation of Hsp90. An increased expression of PTGES3/p23 has been demonstrated to be involved in tumor progression and a poor prognosis in breast cancer (49). Additionally, in acute myelogenous leukemia, PTGES3/p23 was demonstrated to be the target for caspase in chemotherapy-induced apoptosis. The 17 kDa product from the cleavage of PTGES3/p23 by caspase is stable in the apoptotic cells leading to chaperone activity of PTGES3/p23 (50). The results from the present study indicate that PTGES3/p23 is downregulated subsequent to treatment with apigenin, so this protein may participate in the HuCCA-1 cellular apoptosis induced by apigenin.

In conclusion, apigenin, a nutraceutical present in several vegetables and fruits, demonstrated the cytotoxic effect toward HuCCA-1. Apoptotic cell death was detected using two different methods, a flow cytometric analysis (Muse Cell Analyzer) with Annexin-V and dead cell assay kit, and DNA fragmentation confirmed the occurrence of early and late apoptosis. The proteins most significantly altered subsequent to treatment with apigenin were associated with apoptosis. The cleavage of cytokeratin 8, 18 and 19 and the high expression of S100-A6 and S100-A11 indicate that apoptosis was induced by apigenin via a caspase-dependent pathway. A marked reduction in the expression of hnRNP A2/B1 was also observed, possibly with changes of splicing forms, since it has been identified that the binding of apigenin to hnRNP A2/B1 resulted in changes of the splicing forms. The present study aimed to contribute to the understanding of the usefulness of dietary flavonoids such as apigenin.

**Acknowledgements**

The present study was supported by the Chulabhorn Research Institute (grant no. BC 2008-02).

**References**