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Apigenin inhibits hepatoma cell growth through alteration of gene expression patterns

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ABSTRACT

Apigenin, a common plant flavonoid, has been shown to possess anti-tumor properties; however, the underlying molecular mechanisms are still not completely understood. In the present study, we investigated the effects of apigenin on human hepatoma Huh7 cell proliferation, cell cycle distribution, apoptosis, and colony formation *in vitro*, as well as on the tumorigenicity of Huh7 cells *in vivo*. To get more insight into the mechanism of apigenin action, we performed genome-wide expression profiling of apigenin-treated Huh7 cells using cDNA microarrays (Agilent Whole Human Genome Oligo Microarray) that contain 41,000 genes. Ten of the most differentially expressed genes (≥5-fold changes) were selected for further evaluation by quantitative RT-PCR (qPCR) and Western blot analyses. Notably, apigenin (5–20 μg/ml) remarkably inhibited Huh7 cell proliferation and colony formation as compared to the vehicle control, which was in a dose-dependent manner. Accompanying with the decreased growth, apigenin-treated cells showed a cell cycle arrest at G2/M phase and an increased rate of apoptosis. Moreover, the xenografts derived from Huh7 cells were significantly ($p < 0.05$) retarded by the delivery of apigenin (50 μg/mouse/day) relative to the control counterparts. Gene expression profile analysis revealed that 1336 genes were up-regulated and 428 genes were down-regulated by apigenin. The down-regulation of interleukin-4 receptor and ubiquitin specific protease 18 and the up-regulation of SLC27A3 and chemokine (C–C motif) receptor 2 were further confirmed by the qPCR and Western blot results. In conclusion, apigenin exhibits inhibitory effects on hepatoma cell growth, which is likely mediated through alteration of gene expression profiles.

Introduction

Flavonoids have recently attracted much research interest due to their anti-cancer potentials (Kim et al. 2009; Wang et al. 2010). Their chemopreventive activities have been shown in multiple human malignancies (Khan et al. 2008; Salmela et al. 2009). Apigenin (4′,5,7-trihydroxyflavone), one of the most common flavonoids, is widely distributed in fruits and vegetables, such as onions, orange, tea, chamomile and wheat sprouts (Zheng et al. 2005). This flavonoid has been found to possess anti-inflammatory, anti-cancer, and free radical scavenging properties (Khan and Sultana 2006). Apigenin has exhibited strong cytotoxic activity in numerous types of cancer cells (Lu et al. 2010; Kachadourian and Day 2006; Kaur et al. 2008; Lee et al. 2008; Siddique et al. 2008; Choi and Kim 2009; Franzen et al. 2009; Karmakar et al. 2009; Shukla and Gupta 2009; Tan et al. 2009; Zhang et al. 2009), including hepatocarcinogenesis (Singh et al. 2004), neuroblastoma (Karmakar et al. 2009), breast cancer (Lee et al. 2008; Choi and Kim 2009), esophageal squamous cell carcinoma (Zhang et al. 2009), colon cancer (Lu et al. 2010; Kaur et al. 2008), lung cancer (Kachadourian and Day 2006; Tan et al. 2009), and prostate cancer (Franzen et al. 2009; Shukla and Gupta 2009) cells. Impairment of cell mitosis (Chiang et al. 2006) and promotion of cell apoptosis (Chiang et al. 2006; Khan and Sultana 2006; Choi et al. 2007; Patel et al. 2007) haven been proposed to account for the anti-cancer effects of apigenin. Apigenin is capable of coordinating multiple survival-related pathways, including nuclear factor-κB (NF-κB; Gupta et al. 2002), insulin-like growth factor (Shukla et al. 2005; Shukla and Gupta 2009), focal adhesion kinase (Hu et al. 2008; Franzen et al. 2009), beta-catenin (Shukla et al. 2007), phosphatidylinositol 3-kinase (PI3K)/Akt, and p38 (Shukla and Gupta 2007; Kaur et al. 2008; Lee et al. 2008) pathways.

Although extensive study of the apigenin action has been made in a variety of tumor cells, relatively little is known about the molecular mechanisms for the apigenin function in hepatoma cells. In this study, we sought to explore the effects of apigenin on human hepatoma Huh7 cell growth both *in vitro* and *in vivo*. To better

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understand the mechanism for the apigenin action, cDNA microarray hybridization, quantitative reverse transcription-polymerase chain reaction (qPCR), and Western blot analysis were performed to identify potential genes involved in the apigenin function.

Materials and methods

Cell line and cell culture

Human hepatoma Huh7 cell line characterized by p53 mutation with A:T → G:C at codon 220 was purchased from Institute of Cellular Research, Chinese Academy of Science, Shanghai, China. Cells were cultured in Dulbecco's minimum essential medium (DMEM, Invitrogen Inc., Carlsbad, CA, USA), supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified incubator containing 5% CO_2.

Cell proliferation assay

Huh7 cells (1.5×10^3 cells per well) were plated in 96-well plates and cultured for 24 h prior to the treatment with apigenin (>95% of purity; Sigma Chemical Co., St. Louis, MO, USA). Apigenin was dissolved in dimethylsulfoxide (DMSO; Solarbio Science & Technology Co., Ltd., Beijing, China) and made up with the culture medium so that the final concentration of the vehicle was not >0.1%. Cells were incubated with different concentrations of apigenin (5, 10 and 20 μg/ml) or 0.1% DMSO as a negative control. Cells were harvested 1–6 days after the treatments and cell viability was determined by modified MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay with Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc.) according to the manufacturer's instructions. Absorbance was measured at 490 nm and background absorbance measured at 630 nm was subtracted. Each growth curve showed the means and standard deviation (SD) of at least three independent experiments. The growth inhibition was determined using: growth inhibition = (control O.D. − sample O.D.)/control O.D. The IC_{50} value was defined as the concentration of compound that produced a 50% reduction of cell viability.

Colony formation on soft agar

Detailed experimental procedures have been described previously (Wang et al. 2003). Briefly, control or apigenin-treated Huh7 cells were seeded in 6-well plates at a density of 1000 cells per well in regular culture medium. After 10 days, cells were washed with PBS, fixed in 10% methanol for 15 min, and stained in Giemsa for 20 min. Colonies that consisted of >50 cells were scored. Each experiment was repeated at least three times.

Cell cycle and apoptosis analysis

Huh7 cells seeded in 6-well plates at a density of 10^5 cells per well were treated with increasing concentrations of apigenin (5, 10 and 20 μg/ml) or 0.1% DMSO for 48 h. After the treatments, cells were harvested and fixed in 70% ethanol overnight at −20 °C. Following washing with ice-cold PBS, cells were resuspended in 300 μl PBS containing 0.5 mg/ml of propidium iodide (PI) and 0.1 mg/ml of RNase A (200 KU, Calbiochem, San Diego, CA), and incubated at room temperature for a minimum duration of 30 min prior to flow cytometry analysis of DNA content. Cell apoptosis was examined using an annexin V detection kit (Caltag-Medsystems Ltd., Buckingham, UK) according to the manufacturer's instructions. Data acquisition and analysis were done in a FACSort Cytometer (FACSCA, New York). A minimum of 1×10^5 cell events were acquired for each analysis, and each experiment was replicated at least three times.

Tumorigenicity in nude mice

Five-week-old female BALB/c nude mice (16–18 g) were purchased from Experimental Animal Center of Shanghai (Chinese Academy of Sciences, Shanghai, China). For tumorigenicity assays, mice were randomly subdivided into 2 groups ($n = 4$ for each group), and each mouse was injected subcutaneously at a single site with 2×10^6 untreated Huh7 cells. In accordance with the routes of administration described previously (Kaur et al. 2008), apigenin (50 μg/mouse/day) was delivered intraperitoneally within 2 days after the cell inoculation. Mice were killed at 30 days following the cell administration, and the number of tumors as well as their diameters and wet weights was determined for each mouse. The histology of the tumors was examined by hematoxylin and eosin (H&E) staining. All experimental manipulations were undertaken in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Experimental Animal Ethical Committee of Shanghai University of Traditional Chinese Medicine of China.

cDNA microarray expression analysis

Total RNA from three independent cultured Huh7 cells treated with 10 μg/ml apigenin or 0.1% DMSO (control) for 48 h was isolated using TRIzol (Invitrogen Corp.) and the RNeasy kit (Qiagen) according to manufacturer's instructions, including a DNase digestion step. RNA quantity and purity were determined using the ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and denaturing gel electrophoresis. The samples were amplified and labeled using the Agilent Quick Amp labeling kit and hybridized with Agilent Whole Human Genome Oligo Microarray (Agilent, Santa Clara, CA, USA) consisting of 41,000 probes for human genes in Agilent's SureHyb Hybridization Chambers. Microarray experiment was performed in triplicate according to the manufacture protocol. In brief, 2 μg of total RNA was reverse-transcribed to complement DNA (cDNA), labeled with Cy3 dye, and subjected to one-color hybridization. After hybridization and washing, the processed slides were scanned with the Agilent DNA microarray scanner (part number G2505B) using settings recommended by Agilent Technologies. The resulting text files extracted from Agilent Feature Extraction Software (version 9.5.3) were imported into the Agilent GeneSpring GX software (version 7.3 or later) for further analysis. The microarray datasets were normalized in GeneSpring GX using the Agilent FE one-color scenario (mainly median normalization). The positive effect of this median normalization is illustrated in Box-plot (see Supplementary Fig. 1), and genes marked present ("All Targets Value") were chosen for data analysis. Finally, a fold change analysis was carried out by calculating the ratio between the treatment and the control to identify differentially expressed genes. A cutoff value of 2-fold change was used. Genes with expression levels that differed by at least 5-fold from the mean in at least one sample were selected for further evaluation. The gene expression profiling data complied with the Minimum Information About Microarray Experiments (MIAME) standard (Brazma et al. 2001). Microarray experiment was completed by Shanghai KangChen Bio-tech Company, Shanghai, China.

Quantitative reverse transcription-polymerase chain reaction (qPCR)

Following the incubation for 48 h with 10 μg/ml apigenin or 0.1% DMSO, Huh7 cells were harvested for the qPCR experiment. Total RNA was purified using an RNeasy Mini Kit (Qiagen, Valencia, CA), and 4 μg RNA was reverse-transcribed using the Superscript First Strand synthesis system (Invitrogen, Carlsbad, CA) to cDNA. qPCR amplification was performed using SYBR-green detection of

Table 1
Primers used for qPCR analysis.

Genbank ID	Gene name	Forward primer	Reverse primer
NM_024330	SLC27A3	AGAACTTGCCACCTTATGCC	CTCATTTGCCATCCGAACTT
NM_024330	FATP-3	CAGAGACCTTCAAACAGCAGAAAGT	CAGAACGTACAGTGGGTCAGACA
NM_004561	OVOL1	ACAGACCCCCAGAGCAGAG	GACTGTCCCCAAGGGTCAC
NM_000647	CCR2	GTGTGTGGAGGTCCAGGAGT	CAACCCAGCTGGAGTCTCTC
NM_018098	ECT2	GCGTTTTCAAGATCTAGCATGTG	CAATTTTCCCATGGTCTTATCC
NM_000418	IL-4Ra	GAC CTG GAG CAA CCC GTA TC	CAT AGC ACA ACA GGC AGA CG
NM_017414	USP18	CAGACCCTGACAATCCACCT	AGCTCATACTGCCCTCCAGA
NM_000452	SLC10A2	ACGCAGCTATGTTCCACCATC	GCGGGAAGGTGAATACGACA
NM_003959	HIP1R	AGCCTCCGACATGCTGTACT	GTCGTCCTTCACAGACCCAT
NM_138285	NUP35	CTACTCCTGGAACAGGGCAA	TCCGAGCCTGCAGTTTAGAT
NM_002046.3	GAPDH	GTTGGAGGTCGGAGTCAACGGA	GAGGGATCTCGCTCCTGGAGGA

PCR products in real time with an ABI-7500 Sequence Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer's instruction. Primers used for the qPCR analysis were presented in Table 1. The PCR program was initiated by 10 s at 95 °C before 40 thermal cycles, each of 5 s at 95 °C and 34 s at 60 °C. Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) and were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression in each sample. Melting curves for each PCR reaction were generated to ensure the purity of the amplification product. A no-template negative control was also included in each experiment. Analyses of all samples were carried out in triplicate, and the mean values were calculated.

Antibodies and Western blot analysis

Control or apigenin-treated Huh7 cells with were collected at 48 h after the treatments. Total cell lysates were prepared and 30 μg protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by immunoblot analysis. The primary antibodies included: anti-IL-4Rα (sc-28361, Santa Cruz; 1:500), anti-USP18 (LS-B1182-50, LifeSpan BioSciences, Seattle, USA; 1:1000), anti-SLC27A3 (H00011000-M09, Abnova Corp.; 1:1000), CCR2 (ab32144, Abcam, UK; 1:500) and anti-GAPDH (sc-47724, Santa Cruz; 1:2000). Anti-rabbit or anti-mouse secondary antibodies conjugated with horseradish peroxidase were used (Pierce Chromatography Cartridges, USA). The immunoreactive protein was detected using the Western blotting enhanced chemiluminescence (ECL) kit (Santa Cruz), and quantitated using Fluor-S MultiImager and Quantity-One software (Bio-Rad).

Statistical analysis

The results were expressed as mean ± SD. Differences between experimental groups were evaluated by Student's *t*-test. A p value < 0.05 was considered statistically significant.

Results

Apigenin significantly inhibits Huh7 cell growth

To examine the effect of apigenin on the proliferation of hepatoma cells, we treated Huh7 cells with DMSO (control) or various concentrations of apigenin. Within 6 days of culture, apigenin exhibited a marked growth inhibitory effect on Huh7 cells in a dose-dependent manner (Fig. 1A). The IC_{50} was approximately 10.5 ± 0.3 μg/ml (Fig. 1B).

To investigate whether apigenin affected the ability of Huh7 cells to form colonies in soft agar, the same number of viable cells treated with apigenin or DMSO were seeded at low cell density in agar. After 10 days, the existing colonies were visualized and counted microscopically. As shown in Fig. 2A and Table 2, both the numbers and the sizes of colonies were remarkably decreased in the apigenin groups when compared to the control group (p < 0.01).

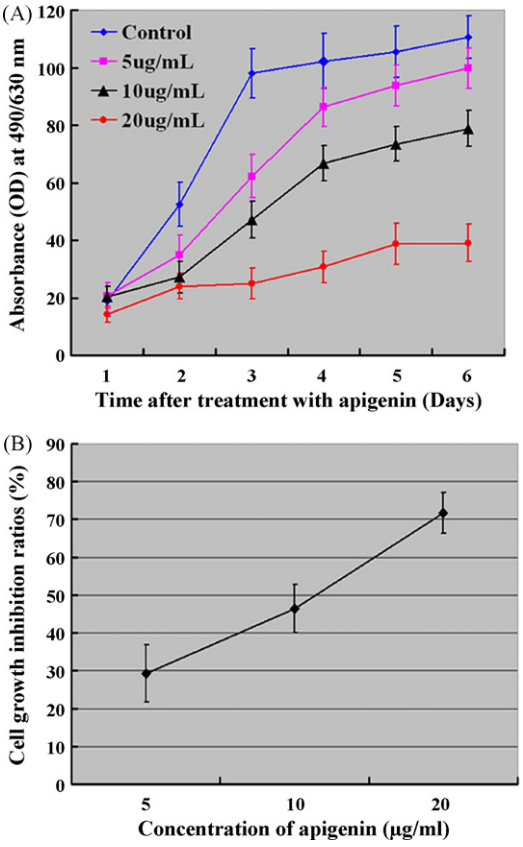

Fig. 1. Growth inhibitory effect of apigenin on Huh7 cells *in intro*. (A) Cells were treated with different concentrations of apigenin for 6 days and cell viability was determined by the MTT assay. (B) The growth inhibition was calculated as percentage of inhibition compared with the control (0.1% DMSO). The cells were treated with apigenin for 72 h. The IC_{50} value was determined graphically from the concentration response curve.

Apigenin induces G2/M cell cycle arrest and apoptosis

The effect of apigenin on cell cycle distribution was analyzed by flow cytometry. As shown in Fig. 2B and Table 3, the treat-

Table 2
The colony formation of Huh7 cells with indicated treatments.

Groups	% colony formation	p
Control (0.1% DMSO)	72.33 ± 15.82	
Apigenin (5 μg/ml)	33.67 ± 6.51	<0.001
Apigenin (10 μg/ml)	24.33 ± 6.51	<0.001
Apigenin (20 μg/ml)	17.33 ± 6.66	<0.001

Table 3
Effect of apigenin on cell cycle progression and apoptosis in Huh 7 cells.

Treatment	Phase of cell population (%)			Apoptotic index
	G0/G1	S	G2/M	
Control (0.1% DMSO)	45.45 ± 5.11	32.45 ± 2.11	22.10 ± 3.07	4.42 ± 1.33
Apigenin (5 μg/ml)	25.20 ± 3.76**	33.51 ± 5.66	41.30 ± 1.90**	11.92 ± 3.03*
Apigenin (10 μg/ml)	23.5 ± 2.53**	28.68 ± 5.09	47.82 ± 2.58**	16.50 ± 3.86**
Apigenin (20 μg/ml)	9.36 ± 3.35**	29.17 ± 1.79	61.47 ± 5.10**	22.34 ± 5.96**

* $p < 0.05$ significantly different from the control group.
** $p < 0.01$, significantly different from the control group.

ment with apigenin caused an increment of G2/M cell population and a concomitant decrement of cell number at G0/G1 phase, and the effect on G2/M arrest was dose-dependent. Moreover, the apigenin treatment resulted in an appearance of a sub-G1 population (apoptosis), which, however, was undetectable in the control group (Fig. 2B). The effect of apigenin on apoptosis was further assessed by PI and annexin V-staining. Consistently, the exposure of Huh7 cells to apigenin substantially increased cell apoptosis as compared with the control group (Fig. 2C and Table 3).

Apigenin suppresses tumorigenicity of Huh7 cells in nude mice

Next, we determined the effect of apigenin on tumorigenicity of Huh7 cells *in vivo*. In accordance with the findings *in vitro*, apigenin administration impaired tumor growth as evidenced by considerably diminished tumor volume in comparison with the control mice ($p < 0.05$; Fig. 3A and B). Microscopically, the tumor cells were arranged in diffuse compact trabeculae, with variable degrees of anaplasia, and increased mitotic activity in the control group. In contrast, obvious necrosis was detected in the apigenin group.

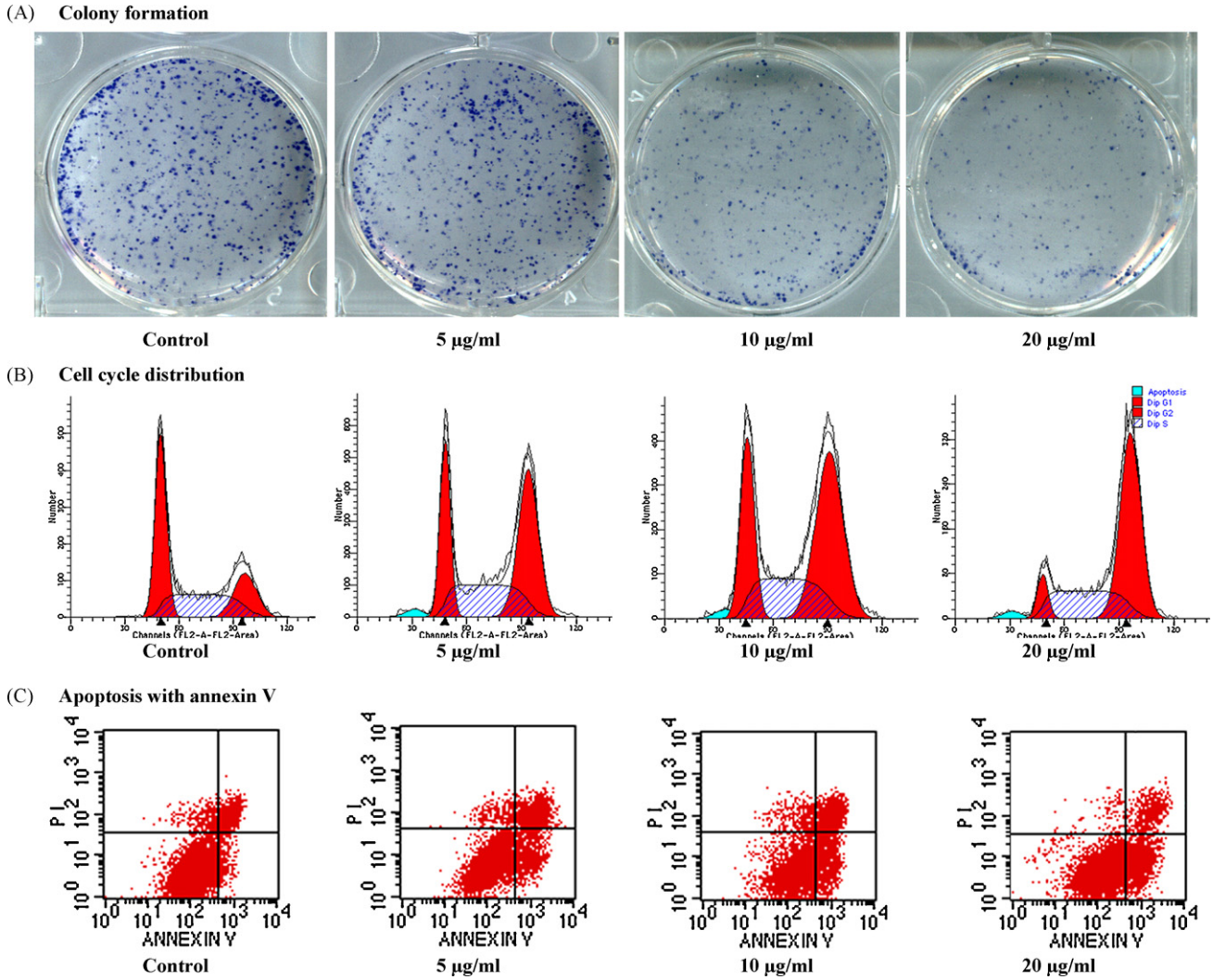

Fig. 2. Effects of apigenin on the colony formation, cell cycle progression and apoptotic indexes in Huh7 cells. (A) Colony formation; (B) Cell cycle distribution and (C) apoptosis analysis of Huh7 cells after culturing with apigenin for 48 h. Treatment with DMSO (0.1%) was used as the control.

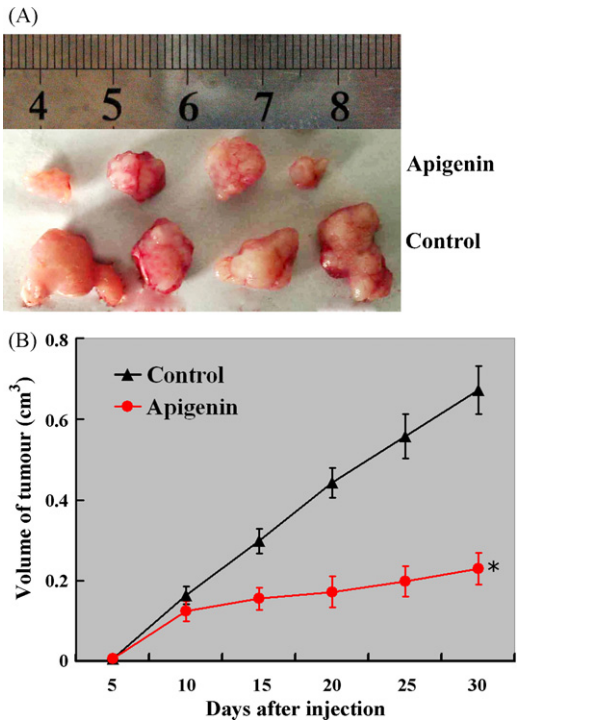

Fig. 3. Effects of apigenin on the tumorigenicity in Huh7-inoculated nude mice. (A) Representative tumor masses from both apigenin (50 μg/mouse/day)-treated and control (0.1% DMSO) groups. (B) Tumor growth curve of each group ($n = 4$). The tumor volumes in the apigenin-treated group were significantly lower than those in the control group ($^{*}p < 0.05$).

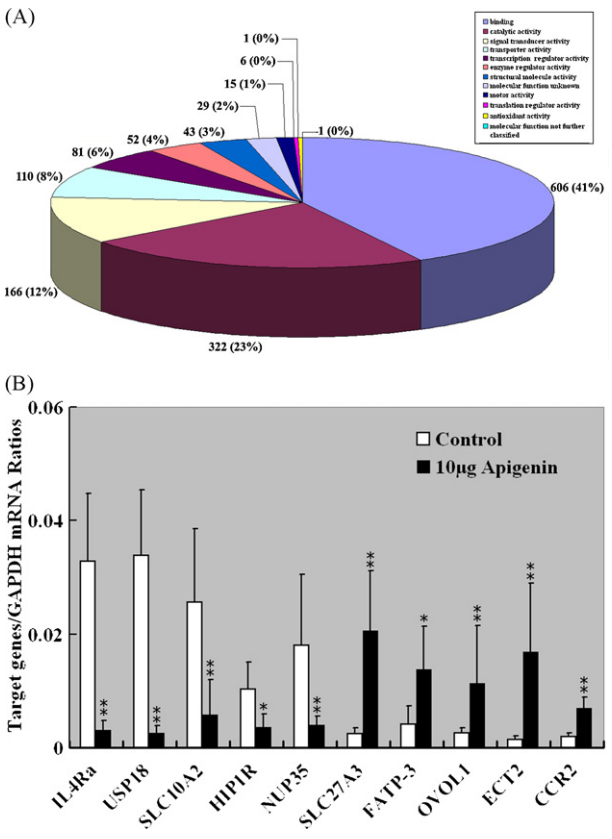

Fig. 4. (A) Bar graphs showing the profile of the differentially expressed genes in Huh7 Cells treated with 10 μg/ml apigenin based on the microarray data. The number (percentage) of each group of differentially expressed genes is shown. (B) The confirmation of the microarray data by qPCR analysis of 10 genes. $^{*}p < 0.05$ and $^{**}p < 0.01$, compared with the control (0.1% DMSO) group.

Apigenin influences genes expression profiles assessed by microarray analysis

To better understand the molecular mechanisms responsible for the inhibitory effects of apigenin, the gene expression profiles of the treated Huh7 cells were analyzed using whole-genome cDNA microarrays. The array results demonstrated that a total of 1764 genes had altered expression levels (>2-fold) between apigenin-treated and control cells; i.e., 1336 genes were up-regulated and 428 genes were down-regulated. Most of these differentially expressed genes are involved in gene expression regulation, cytoskeletal structure and/or adhesion, signal transduction, metabolism, apoptosis or immune responses (Fig. 4A).

Confirmation of differential levels of gene expression by qPCR and Western blotting

The genes with expression altered by more than 5-fold were summarized in Table 4. To verify the microarray data, qPCR analysis was conducted for 10 selected genes. The qPCR and microarray expression results matched well, although there was variation in fold change for gene expression. Notably, IL-4R and USP18 mRNA levels were markedly decreased in apigenin-treated cells as compared with the control cells, whereas, conversely, SLC27A3 and chemokine (C–C motif) receptor 2 (CCR2) expression were remarkably up-regulated in the cells treated with apigenin (Fig. 4B). Western blot analysis further confirmed the gene expression alterations revealed by qPCR (Fig. 5A and B).

Discussion

Apigenin, one of the most abundant natural flavonoids, has been shown to possess anti-tumor activity in a variety of tumor cells (Lu et al. 2010; Kachadourian and Day 2006; Kaur et al. 2008; Lee et al. 2008; Siddique et al. 2008; Choi and Kim 2009; Franzen et al. 2009; Karmakar et al. 2009; Shukla and Gupta 2009; Tan et al. 2009; Zhang et al. 2009). In this study, we consistently demonstrated that apigenin inhibits hepatoma cell growth both *in vitro* and *in vivo*, which is coupled with induction of G2/M arrest. Moreover, apigenin treatment causes significant apoptosis of hepatoma cells compared to the control treatment. The inhibitory effects of apigenin on hepatoma cells may be attributable to its coordination of a large number of effector genes. Particularly, two tumorigenesis-related genes IL-4R and USP18 were found to be remarkably down-regulated at both mRNA and protein levels by apigenin. Previous studies have demonstrated that IL-4R are overexpressed on the surface of various human solid tumors including glioblastoma, lung, ovarian, and head and neck squamous cell carcinoma (Kioi et al. 2005; Melstrom et al. 2008). In tumorigenesis, IL-4R activates several signaling pathways, including p38-MAPK, PI3-kinase/Akt, and JAK-STAT signal pathway (Puri et al. 2009). These signaling pathways have also been shown to be regulated by apigenin in numerous types of cancers, ultimately controlling cancer cell growth (Shukla and Gupta 2007; Shukla et al. 2007; Hu et al. 2008; Kaur et al. 2008; Lee et al. 2008; Franzen et al. 2009; Shukla and Gupta 2009). Inhibition of IL-4R signaling has been proposed as a novel treatment for cancers (Kioi et al. 2005; Todaro et al. 2008). These findings suggest that down-regulation of IL-4R may partially account for the inhibitory effects of apigenin in hepatoma cells. Interestingly, our unpublished data of qPCR and Western blot analyses reveal that the overexpression of IL-4R is observed in 17 of 20 human hepatocellular carcinoma (HCC) specimens, thus implying a potential role of apigenin in treatment of HCC.

Table 4
Genes differentially expressed after apigenin treatment in Huh7 cells.

Genbank ID	Gene symbol	Genes title	Description	Cytoband	Fold change
NM_024330	SLC27A3	Homo sapiens solute carrier family 27 (fatty acid transporter), member 3	Metabolism; catalytic activity	1q21.3	27.69 ± 0.24
NM_004561	OVOL1	Homo sapiens ovo-like 1(Drosophila)	Regulation of transcription, DNA-dependent	11q13	6.22 ± 0.74
	A_32_P100452		Unknown function		6.05 ± 0.28
	THC2376306	Q7QRW0 GLP_69_6195_6536, partial (14%)	Unknown function		5.99 ± 0.45
NM_000647	CCR2	Homo sapiens chemokine (C–C motif) receptor 2 (CCR2), transcript variant A	Chemotaxis; inflammatory response	3p21.31	5.98 ± 0.40
	THC2355348	ALU1_HUMAN (P39188) Alu subfamily J sequence contamination warning entry, partial (7%)	Unknown function		5.90 ± 0.49
NM_018341	C6orf70	Homo sapiens chromosome 6 open reading frame 70	Unknown function	6q27	5.77 ± 0.59
NM_018098	ECT2	Homo sapiens epithelial cell transforming sequence 2 oncogene	Intracellular signaling cascade; positive regulation of I-kappaB kinase/NF-kappaB cascade	3q26.1–q26.2	5.61 ± 0.67
NM_032109	OTP	Homo sapiens orthopedia homolog (Drosophila)	Regulation of transcription, DNA-dependent	5q13.3	5.53 ± 0.59
AK055101	C6orf168	Chromosome 6 open reading frame 168	Unknown function	6q16.2–q16.3	5.37 ± 0.87
NM_006552	SCGB1D1	Homo sapiens secretoglobin, family 1D, member 1	Unknown function		5.27 ± 0.75
NM_000918	P4HB	Homo sapiens procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide (protein disulfide isomerase-associated 1)	Protein disulfide isomerase activity; procollagen-proline 4-dioxygenase activity	17q25	5.20 ± 0.67
BC049371	HERPUD2	Homo sapiens HERPUD family member 2	Protein modification	7p14.2	5.20 ± 0.66
NM_144578	MAPK1IP1L	Homo sapiens mitogen-activated protein kinase 1 interacting protein 1-like	Signaling pathway	14q22.3	5.09 ± 0.82
NM_016353	ZDHHC2	Homo sapiens zinc finger, DHHC-type containing 2	Protein palmitoylation, tumor suppressor	8p21.3–p22	5.09 ± 0.72
NM_004329	BMPR1A	Homo sapiens bone morphogenetic protein receptor, type IA	Protein amino acid phosphorylation, transforming growth factor beta receptor signaling pathway	10q22.3	5.08 ± 0.52
NM_001144	AMFR	Homo sapiens autocrine motility factor receptor (AMFR), transcript variant 1	Cell motility; signal transduction; protein ubiquitination	16q21	5.03 ± 0.69
NM_000418	IL-4R	Interleukin-4 receptor (IL-4R), transcript variant 1	Immune response; signal transduction	16p12.1–p11.2	0.18 ± 0.08
BC070363	BC070363	Homo sapiens cDNA clone IMAGE:3960708, partial cds	Unknown function		0.19 ± 0.02
NM_017414	USP18	ubiquitin specific protease 18	Ubiquitin-dependent protein catabolism	22q11.21	0.19 ± 0.04
NM_000452	SLC10A2	Solute carrier family 10 (sodium/bile acid cotransporter family), member 2	Ion transport	13q33	0.19 ± 0.07
AK026477	AK026477	Homo sapiens cDNA: FLJ22824 fis, clone KAIA3991	Unknown function	15q25.2	0.19 ± 0.07
NM_003959	HIP1R	Huntingtin interacting protein-1-related (HIP1R), mRNA	Biological_process	12q24	0.19 ± 0.08
NM_138285	NUP35	Nucleoporin 35kDa, transcript variant 1	mRNA and protein transport	2q32.1	0.19 ± 0.04
NM_000170	GLDC	Glycine dehydrogenase (decarboxylating; glycine decarboxylase, glycine cleavage system protein P)	Glycine catabolism	9p22	0.19 ± 0.03

Note: The data represent mean ± SD in triplicate. Huh7 cells treated with 0.1% DMSO for 48 h are used as the control.

USP18, a member of the ubiquitin specific protease family (Zou et al. 2007), is another down-regulated gene. Recent evidence indicates that knockdown of USP18 causes an increased apoptosis of chronic myeloid leukemia BCR-ABL+ KT-1 cells upon interferon-α treatment (Lu et al. 2010). This gene is involved in the modulation of multiple signaling pathways in normal and cancer cells (Randall et al. 2006; Duex and Sorkin 2009; Sarasin-Filipowicz et al. 2009). A previous study (Kim et al. 2008) has documented that down-regulation of USP18 can disrupt HBV infection that is a well-established risk factor for HCC. These observations suggest that apigenin may have implications in the prevention of virus infection and HCC development.

In addition, apigenin treatment has an influence on the genes involved in metabolism (SLC27A3, P4HB and GLDC), DNA-dependent regulation of transcription (OVOL1 and OTP), cell signal transduction (ECT2, MAPK1IP1L, BMPR1A and AMFR), cell motility (AMFR), inflammatory response (CCR2) and other biological processes (HIP1R, ZDHHC2, NUP35 and SLC10A2). We also found that a gene, whose probe set ID is A_32_P100452 at in Agilent whole-genome oligonucleotide microarray but with unknown function, is up-regulated by about 6-fold. Further functional studies are needed to assess the role of the array-identified genes in the apigenin-mediated anti-cancer activity.

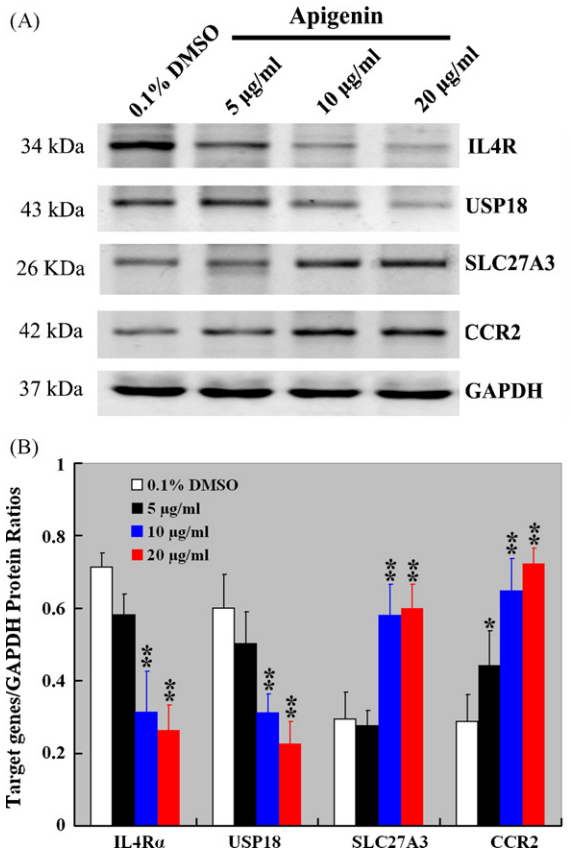

Fig. 5. Western blot analysis of the IL-4R, USP18, SLC27A3 and CCR2 protein levels. (A). Huh7 cells were treated with control (0.1% DMSO) or indicated concentrations of apigenin for 48 h and subjected to Western blot analysis. Representative bands were shown. (B) Bar graphs showing the quantification of Western blot bands. GAPDH was used as an internal control. $^{*}p < 0.05$ and $^{**}p < 0.01$, compared with the control (0.1% DMSO) group.

In conclusion, our results demonstrate an inhibitory role for apigenin in hepatoma cells, which is associated with induction of G2/M arrest and apoptosis. Alteration of gene expression patterns and especially down-regulation of IL-4R and USP18 may account for the inhibitory activity of apigenin.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2010.08.006.

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