Characterization of gene expression regulated by American ginseng and ginsenoside Rg3 in human colorectal cancer cells

XIAOJI LUO[1,2], CHONG-ZHI WANG[3], JIN CHEN[1,2], WEN-XIN SONG[1], JINYONG LUO[1,2], NI TANG[1,2],
BAI-CHENG HE[2], QUAN KANG[2], YITAO WANG[4], WEI DU[5], TONG-CHUAN HE[1,2] and CHUN-SU YUAN[3]

[1]Molecular Oncology Laboratory, Department of Surgery, The University of Chicago, Chicago, IL, USA; [2]Key Laboratory
of Diagnostic Medicine designated by Chinese Ministry of Education and the Affiliated Children's Hospital of Chongqing
Medical University, Chongqing, P.R. China; [3]Tang Center for Herbal Medicine Research, The University of Chicago,
Chicago, IL, USA; [4]Institute of Chinese Medical Sciences, University of Macau, Taipa, Macau, P.R. China;
[5]Ben May Department of Cancer Research, The University of Chicago, Chicago, IL, USA

Abstract. American ginseng (*Panax quinquefolius* L., Araliaceae) possesses anti-cancer potential and is one of the most commonly used herbal medicines in the United States. Ginsenoside Rg3, one of the saponins in American ginseng, has been shown to inhibit tumor growth. In this study, we sought to characterize the downstream genes targeted by American ginseng extracts in HCT-116 human colorectal cancer cells. We first demonstrated that the content of Rg3 in American ginseng steamed at 120˚C for 2 h (referred to as S2h) was significantly increased when compared with that of the unsteamed ginseng. Both S2h and Rg3 exhibited anti-proliferative effects on HCT-116 cells. Using the Affymetrix high density genechips containing more than 40,000 genes and ESTs, the gene expression profiling of HCT-116 cells were assayed. Microarray data indicated that the expression levels of 76 genes were changed significantly after treatment with S2h or Rg3, whereby it was found that 52 of the 76 genes were up-regulated while the remaining 24 were down-regulated. Ingenuity Pathways Analysis of top functions affected by both S2h and Rg3 were carried out. The most effected pathway is the Ephrin receptor pathway. To validate

Correspondence to: Dr Chun-Su Yuan, Tang Center for Herbal Medicine Research, and Department of Anesthesia & Critical Care, The University of Chicago Medical Center, 5841 South Maryland Avenue, MC 4028, Chicago, IL 60637, USA
E-mail: cyuan@dacc.uchicago.edu

Dr T.-C. He, Molecular Oncology Laboratory, Department of Surgery, The University of Chicago Medical Center, 5841 South Maryland Avenue, MC 3079, Chicago, IL 60637, USA
E-mail: tche@surgery.bsd.uchicago.edu

the microarray data, quantitative real-time PCR of six candidate target genes was conducted, whereby it was found that three genes were up-regulated (AKAPA8L, PMPCB and PDE5A) and three were down-regulated (PITPNA, DUS2L and RIC8A). Although further studies are needed to elucidate the mechanisms of action, our findings should expand the understanding of the molecular framework of American ginseng as an anti-cancer agent.

Introduction

American ginseng (*Panax quinquefolius* L., Araliaceae) an obligate shade perennial plant native to eastern North America, is a commonly used herbal medicine in the US (1). The major active components of American ginseng are ginsenosides, a group of steroidal saponins distributed throughout many parts of the plant, including the root, leaf and berry. A number of investigations have demonstrated anti-cancer properties and other pharmacological activities possessed by Asian ginseng (*Panax ginseng* C. A. Meyer) (2) and notoginseng [*Panax notoginseng* (Burk.) F.H. Chen] (3), two important species in genus *Panax*, whereby ginsenosides Rg3 and Rh2 have been recognized as active anti-cancer compounds (4). Like Asian ginseng (2,5), American ginseng also has anti-cancer activities (6,7). In addition, American ginseng extract was found to enhance the anti-cancer effect of chemotherapeutic agents and attenuate cisplatin-induced nausea and vomiting (8,9). In contrast to the multitude of studies on Asian ginseng's anti-cancer effects, investigations on American ginseng are limited (10) and their mechanisms of action remain unidentified. Moreover, research on the effect of heating American ginseng is rare (11) and the activities and mechanisms of steamed American ginseng on cancer cells have not been studied.

To better understand the anti-cancer activities of the compounds found in American ginseng, it is essential to clarify the mechanisms related to individual molecules. To date, the influences of American ginseng and ginsenoside Rg3 on genomic expression were largely unknown. In this study, microarray hybridization was used to identify genomic

aberrations in HCT-116 human colorectal cancer cells treated with steamed American ginseng extract (S2h) and ginsenoside Rg3 in order to explore the potential mechanisms of the anti-cancer effect. The results suggest that ginsenoside Rg3 and S2h can inhibit the growth of HCT-116 cells significantly. The anti-cancer mechanism of American ginseng possibly involves the up-regulated gene of A-kinase (PRKA) anchor protein 8-like (AKAPA8L) and the down-regulated gene of phosphatidylinositol transfer protein alpha (PITPNA).

Materials and methods

Chemicals and preparation of steamed American ginseng extract S2h. Ginsenoside standards for Re, Rb1, Rc, Rd and Rg3, were obtained from the Delta Information Center for Natural Organic Compounds (Xuancheng, Anhui, China). All standards were of biochemical-reagent grade and at least 95% pure as confirmed by HPLC. For the heat-processing of American ginseng, the roots were steamed at 120˚C (AMSCO 2022 Autoclave, AMSCO Scientific, Apex, NC, USA) for 2 h, and then lyophilized to obtain dried samples. For the analytical and *in vitro* anticancer studies, the American ginseng extracts were prepared as follows. The root samples, unsteamed or steamed at 120˚C for 2 h (S2h), were ground and extracted with 70% ethanol. The solvent of the extract solution was evaporated under vacuum. The dried extract was dissolved in water, and then extracted with water-saturated n-butanol. The n-butanol phase was evaporated under vacuum and then lyophilized.

HPLC analysis. The HPLC system was a Waters 2960 instrument with a 996 photodiode array detector (Milford, MA, USA). The separation was carried out on an Alltech Ultrasphere C18 column (5 μ, 250x3.2 mm I.D.) (Deerfield, IL, USA) with a guard column (Alltech Ultrasphere C18, 5 μ, 7.5x3.2 mm I.D.) (12). Acetonitrile (solvent A) and water (solvent B) were used. Gradient elution started with 18% solvent A and 82% solvent B, and was changed to 21% A for 20 min, and then to 26% A for 3 min and held for 19 min; to 36% A for 13 min; to 50% A for 9 min; to 95% A for 2 min and held for 3 min; and finally changed to 18% A for 3 min and held for 8 min. The flow rate was 1.0 ml/min and the detection wavelength was set to 202 nm. Ginsenoside standards, unsteamed and steamed American ginseng extracts were dissolved in methanol. All tested solutions were filtered through Millex 0.2-μm nylon membrane syringe filters before use. The contents of saponins in each sample were calculated using standard curves of ginsenosides.

Cell culture and cell proliferation analysis. HCT-116 human colorectal cancer cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in the McCoy's 5A medium supplemented with 10% FBS and 50 IU penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 37˚C. HCT-116 cells were seeded in 96-well plates (1x10^4 cells/well). After 1 day, various concentrations of S2h or Rg3 were added to the wells. The final concentration of ethanol was 0.5%. Controls were exposed to culture medium containing 0.5% ethanol without drugs. All experiments were performed in triplicate and repeated 3 times. Cell proliferation was evaluated using an MTS assay according to the manufacturer's instructions after treatment for 48 h. Data are presented as mean ± standard deviation (SD). A One-way ANOVA determined whether the results had statistical significance.

RNA extraction and quality control. HCT-116 cells were treated with the ginsenoside Rg3 (concentration of 100 μM), steamed American ginseng extract S2h (concentration of 75 μg/ml), and solvent control. Cells were collected at 24 h or 48 h after treatment for total RNA isolation by using Trizol reagent (Invitrogen, USA). The quality of total RNA was evaluated from the following four aspects: quantity (>20 μg), concentration (>1 μg/μl), purity (OD 260/280 ≥1.8), and integrity (intact 18S and 28S ribosomal RNA bands). The first three parameters were measured using a conventional spectrophotometer, and the fourth parameter was evaluated on MOPS/Formaldehyde agarose gels.

Microarray hybridizations and data analysis. Before microarray experiments, RNA quality was confirmed using an Agilent 2100 Bioanalyzer and a GeneSpec III. Fully characterized RNA samples were subjected to hybridizations to Affymetrix human high density genechips U133 plus 2 (totally containing >40,000 known genes and ESTs) that was carried at The Functional Genomics Facility of The University of Chicago. The acquisition and initial quantification of array images were performed using the Affymetrix Microarray Suite Version 5.0 (MAS 5.0) with the default analytic parameters (Alpha 1: 0.04; Alpha 2: 0.06; Tau: 0.015; Global scaling target signal: 500). We used a two-step filtration strategy in order to remove noise while retaining true biological information. The first step was to filter genes with signal intensity in all samples ≤100 intensity units. The second step filtration was to remove the genes that receive an 'absent' call for all chips. All data had been scaled to a target signal of 500 and therefore they were comparable among samples. Results from two different normalization strategies were compared. All the data has been analyzed by DNA-Chip Analyzer (dCHIP) and Ingenuity Pathways Analysis (IPA).

Quantitative real-time PCR (qPCR) analysis. The mRNA level of the candidate genes was validated by quantitative real-time RT-PCR. Briefly, the same RNA samples for microarray were used to generate cDNA templates for reverse transcription reactions (13). The first strand cDNA synthesis was performed using a hexamer (Promega) and Superscript II reverse transcriptase (Invitrogen). The SYBR Green-based qRT-PCR analysis was carried out using the Opticon DNA Engine thermocycler (MJ Research, Waltham, MA). The cycling program was as follows: 94˚C x 2 min for one cycle, 4 cycles at 92˚C x 20 sec, 68˚C x 30 sec, and 72˚C X 20 sec with a decrease of 3 degrees/cycle, and 30 cycles at 92˚C x 20 sec, 57˚C x 30 sec, and 72˚C X 20 sec, followed by a plate read at 75˚C each cycle. Triplicate reactions were carried out for each sample. All samples were normalized by the expression level of glyceraldehyde-3-phosphate dehydrogenase. Specificity of PCR products obtained was characterized by melting curve analysis, followed by gel electrophoresis (Table III).
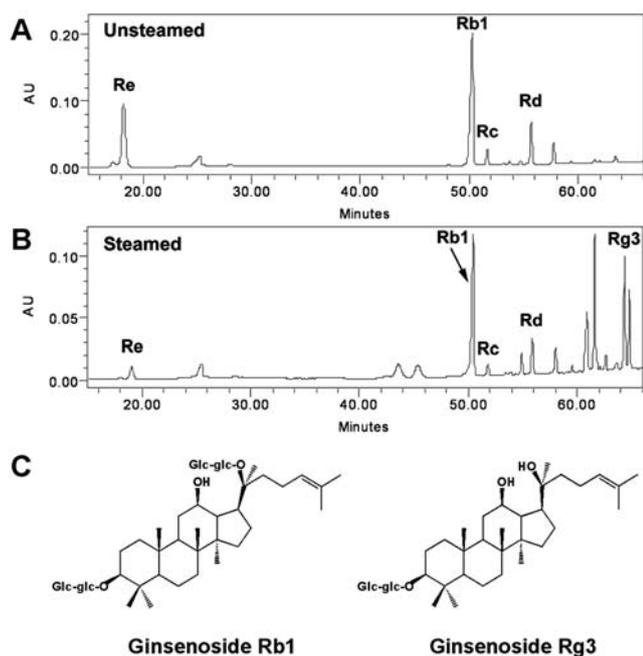
Figure 1. HPLC analysis of ginsenosides in American ginseng. (A) Chromatogram of unsteamed American ginseng. (B) Chromatogram of steamed American ginseng. (C) Chemical structures of ginsenosides Rb1 and Rg3.

Results

Chemical constituent changes during the steaming treatment assayed by HPLC. Five saponins, ginsenosides Re, Rb1, Rc, Rd and Rg3 were determined in American ginseng extracts using high performance liquid chromatography (HPLC). All the assayed ginsenosides were dammarane glycosides (14,15). Chromatograms of American ginseng extracts, unsteamed and steamed, are shown in Fig. 1A and B. The chemical structures of ginsenoside Rb1 and Rg3, which are the main constituents in unsteamed and steamed American ginseng are shown in Fig. 1C. The peak areas of Rb1, Rc, Rd and Re decreased during the steaming process. On the other hand, ginsenoside Rg3, which is a trace saponin in unsteamed root (16), was augmented during the steaming process (Fig. 1A and B). The contents of ginsenosides Re, Rb1, Rc, Rd and Rg3 in unsteamed American ginseng root extract were 19.8, 34.2, 3.4, 6.5 and 0.06%, while in steamed extract S2h were 2.1, 17.3, 1.2, 2.9 and 5.9%, respectively. During the steaming process, the content of ginsenoside Rg3 was increased significantly.

Antiproliferative effects of S2h and ginsenoside Rg3 on HCT-116 cells. The antiproliferative effects of unsteamed and steamed (S2h) American ginseng root extracts on HCT-116 human colorectal cancer cells were assayed by the modified trichrome stain (MTS) method. Along with augmentation of drug concentration, the HCT-116 cells were inhibited by both the extracts. Unsteamed American ginseng extract had antiproliferative effects on the HCT-116 cells, but the effects were minimal at 100-250 μg/ml. S2h had very significant antiproliferative effects at 250 μg/ml (Fig. 2A). Heat-processing increased the antiproliferative effect of American
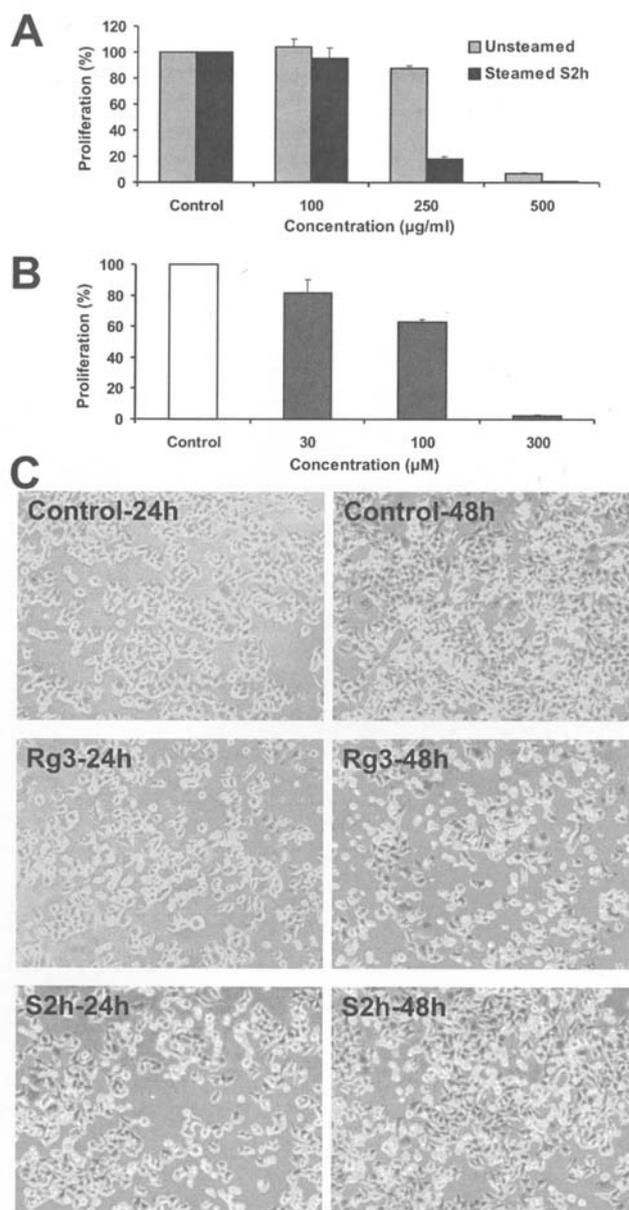

Figure 2. Effects of American ginseng and ginsenoside Rg3 on the proliferation of HCT-116 human colorectal cancer cells. (A) Cells were treated with unsteamed and steamed (S2h) American ginseng extracts for 48 h. (B) Cells were treated with Rg3 for 48 h. (C) Microscopic images of HCT116 cells treated by Rg3 (100 μM) and S2h (75 μg/ml). The treated cells were used for total RNA isolation at the indicated time points and for microarray analysis.

ginseng significantly. As one of the active constituents in American ginseng, the antiproliferative effect of single compound Rg3 has also been evaluated. From the results shown in Fig. 2B, ginsenoside Rg3 at 100-300 μM, showed obvious antiproliferative effects on HCT-116 cells.

The morphologic changes of HCT-116 cells, treated by Rg3 (100 μM) and S2h (75 μM), are shown in Fig. 2C. Under the same treatment conditions, cell death was more apparent at 48 h post treatment. Total RNA was isolated from the treated and control cells and subjected to microarray analysis.

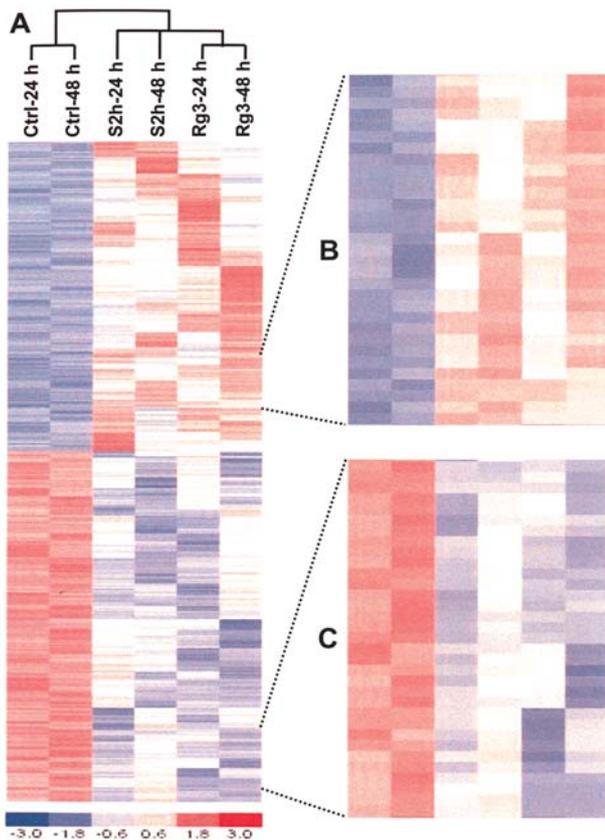*The dChip analysis of microarray data of HCT-116 human colon cancer cells treated with S2h and ginsenoside Rg3.* The

Figure 3. The dChip clustering analysis of the gene expression pattern affected by both Rg3 and S2h (A). The filtered 409 genes (p<0.05) were used for the clustering analysis. (B) Representative gene cluster that was up-regulated by both Rg3 and S2h. (C) Representative gene cluster that was down-regulated by both Rg3 and S2h.

model-based expression analysis using DNA-Chip Analyzer (dCHIP) with the *.CEL files obtained from MAS 5.0 (17) was performed. This commonly-used model-based approach allows probe-level analysis and facilitates automatic probe selection in the analysis stage to reduce errors caused by outliers, cross-hybridizing probes, and image contamination. In this experiment, we selected differentially expressed genes at a fold change of 1.2 (default setting) or 2.0 with a 90% confidence interval constructed using standard errors of expression values. Four characteristics were considered when determining whether a given gene was differentially expressed: a) absolute expression level; b) subtractive degree of change between groups; c) fold change between groups; and d) reproducibility of measurement.

We performed the dCHIP clustering analysis using both Rg3 and S2h treated samples as an experimental group vs. the control treatment group. As shown in Fig. 3, there were 409 genes that satisfied the comparison filtering criteria (fold ≥1.2, p<0.05, i.e. the default setting). Among the 409 genes, the expression of 192 genes was up-regulated by at least 1.2-fold by both Rg3 and S2h (exemplified by Fig. 3B), while the expression of 217 genes was inhibited by at least 1.2-fold by both Rg3 and S2h (exemplified by Fig. 3C). There are subsets of genes that were regulated in a different manner by S2h than by Rg3, while there are some regions displaying a similar expression pattern. To identify the most significantly regulated genes by Rg3 and S2h, we increased the stringency for dCHIP analysis: fold ≥2 and p<0.05. There are 76 genes that satisfied the comparison filtering criteria. Among the 76 genes, 52 genes are up-regulated, while 24

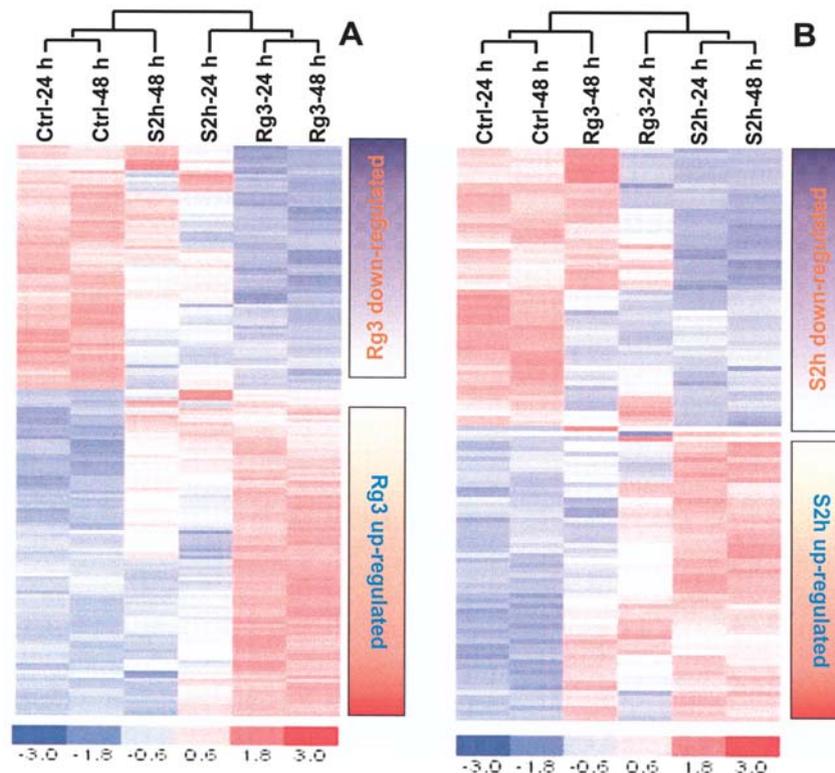Figure 4. The dChip clustering analysis based on both either Rg3 or S2h-regulated gene expression pattern. (A) The clustering analysis of the gene expression pattern regulated by Rg3. The filtered 159 genes (p<0.05) were used for the clustering analysis. (B) The clustering analysis of the gene expression pattern regulated by S2h. The filtered 113 genes (p<0.05) were used for the clustering analysis.

Table I. Top 20 up-regulated genes regulated by both Rg3 and S2h (>2-fold).

Gene and description	Accession no.	Control 24 h	Control 48 h	Rg3 24 h	Rg3 48 h	S2h 24 h	S2h 48 h	Fold Change	P-value
FYN oncogene related to SRC/FGR/YES	N20923	121.0	122.5	282.8	260.7	291.9	278.5	2.29	0.0001
Keratin 85	NM_002283	20.3	14.3	175.0	171.9	196.6	138.8	9.86	0.0006
EST	BF940127	37.6	30.9	171.0	127.7	144.3	127.4	4.16	0.0010
EST	BC020820	37.7	41.0	140.4	180.3	142.9	175.6	4.06	0.0012
ORF	AL122087	50.9	38.0	201.0	180.1	160.9	136.3	3.81	0.0013
Chitinase 3-like 1	AJ251847	124.9	139.2	275.7	299.6	287.7	360.5	2.32	0.0014
EST	BG111938	89.5	107.6	199.7	230.8	207.0	239.7	2.23	0.0022
EST	AK093301	16.3	20.3	126.0	148.6	123.8	92.1	6.70	0.0024
PLUC	NM_016583	38.8	59.0	209.2	169.3	192.1	167.6	3.77	0.0026
Tubulin, beta 1 (TUBB1)	NM_030773	44.2	30.7	178.1	114.7	146.2	180.9	4.14	0.0027
A kinase (PRKA) anchor protein 8 (AKAPAL8)	AW016526	73.2	76.2	197.4	225.6	220.7	159.8	2.69	0.0033
Snf2-related CBP activator protein	AB002307	69.7	88.3	145.8	188.0	188.7	206.0	2.31	0.0034
ORF	NM_025002	38.3	46.6	302.2	314.8	276.3	190.7	6.38	0.0034
Peptidase (mitochondrial processing) beta	AF520796	101.0	86.1	339.0	275.5	254.5	205.8	2.87	0.0060
EST	AI806403	75.8	103.5	227.3	273.9	180.6	195.9	2.45	0.0067
EST	AA668789	81.4	101.8	308.9	184.7	255.4	205.4	2.60	0.0095
ORF	BI827290	43.4	41.1	252.0	201.3	144.3	154.2	4.45	0.0096
Smad6	AI628464	62.1	81.2	262.7	235.7	133.3	222.8	2.98	0.0113
Phosphodiesterase 5A, cGMP-specific	AB015656	58.5	87.6	139.5	221.3	189.4	174.8	2.48	0.0121
Troponin T3, skeletal, fast	BF789882	43.6	62.2	291.1	192.0	338.1	176.2	4.71	0.0128

genes are down-regulated. From this gene list, the top 20 up-regulated genes (Table I) and top 20 down-regulated genes (Table II) are identified, based on the p-value sorting (the lowest to the highest). As shown in Tables I and II, nearly half of the top 20 genes in each category are ESTs or ORFs. However, the lists include several genes that may serve as important targets in Rg3 and S2h-mediated anti-cancer activity in human colon cancer cells. Interestingly, Gene Ontology analysis revealed that genes involved in nucleotide binding was the top category significantly regulated by both Rg3 and S2h.

We conducted further dCHIP analysis by focusing on the genes primarily regulated by either Rg3 or S2h. Under similar analysis conditions as described for Fig. 3, we found that 159 genes were significantly regulated by Rg3, whereas 113 genes were significantly regulated by S2h. The gene expression pattern of these significantly regulated genes was used for clustering analysis and shown in Fig. 4. The Gene Ontology analysis revealed that genes involved in 'steroid hormone receptor activity' and 'ligand-dependent nuclear receptor activity' were among the top categories regulated primarily by Rg3, while the Gene Ontology of S2h-regulated genes was less apparent.

Ingenuity Pathways Analysis microarray data of HCT-116 human colon cancer cells treated with S2h and ginsenoside Rg3. Through the Ingenuity Pathways Analysis (IPA) of microarray, we performed gene network analysis using the significant gene list of 409 from dCHIP analysis. These genes identified as having a recognized function and can be classified into three major groups by IPA.

IPA Network analysis: there are 12 gene networks that were affected, although most of the networks only had a few genes that were differentially affected. One of the most affected gene networks is shown in Fig. 5A, in which all 35 genes of this network were either up-regulated or down-regulated by both Rg3 and S2h in HCT-116 cells.

Top pathways affected by Rg3 and S2h, as shown in Fig. 5B, were the signal pathways, such as Ephrin receptor, TGFß signaling, SAPK/JNK signaling, and PI3K/AKT signaling, are among the most significantly affected functions by both Rg3 and S2h.

Table II. Top 20 down-regulated genes regulated by both Rg3 and S2h (>2-fold).

Gene and description	Accession no.	Control 24 h	Control 48 h	Rg3 24 h	Rg3 48 h	S2h 24 h	S2h 48 h	Fold Change	P-value
Hydroxysteroid (17-beta) dehydrogenase 14	BC006283	301.8	318.1	94.9	137.6	109.0	149.5	-2.53	0.0003
ORF	NM_153755	205.6	210.1	31.9	82.9	58.2	55.6	-3.64	0.0005
EST	AK026465	156.1	160.5	16.2	53.4	43.3	58.4	-3.70	0.0008
ORF	AA043429	172.4	191.6	58.9	56.3	109.3	67.2	-2.50	0.0030
ATP-binding cassette C (CFTR/MRP) 11	BC039085	198.6	176.8	40.7	35.7	101.8	78.9	-2.92	0.0033
ORF	AA002166	146.5	162.4	84.9	17.9	52.3	17.8	-3.57	0.0036
Phosphatidylinositol transfer protein, alpha	AI732782	182.0	159.7	35.1	109.9	39.5	40.0	-3.04	0.0057
Ras homolog enriched in brain 2 (RHEB)	AA056145	290.3	308.2	214.3	98.3	145.6	119.3	-2.07	0.0060
Tetratricopeptide repeat domain 12	BC032355	219.2	248.7	124.8	101.6	72.0	162.5	-2.03	0.0097
EST	AF131796	202.8	199.7	81.9	149.5	94.6	46.5	-2.16	0.0146
Small nuclear ribo-nucleoprotein 70 kDa	AK024127	156.0	175.7	20.2	22.2	73.1	119.2	-2.83	0.0154
Dihydrouridine synthase 2-like SMM1 homolog	NM_017803	1065.1	1035.7	151.1	190.0	766.6	162.0	-3.31	0.0159
EST	AW206465	192.3	226.8	56.2	140.5	42.0	116.4	-2.36	0.0160
RIC8A	AI492888	246.0	284.7	72.1	75.6	68.0	123.9	-3.13	0.0167
ORF	AW119023	254.5	213.2	43.3	57.4	112.1	100.1	-2.99	0.0184
Phosphodiesterase 6B, cGMP-specific	BC000249	224.7	193.8	41.3	41.2	43.1	170.1	-2.83	0.0198
EST	AI634534	136.4	171.0	97.2	21.2	57.2	16.8	-3.20	0.0220
EST	AI076273	203.3	242.3	91.5	60.5	88.0	139.3	-2.35	0.0233
TIRAP	AW956783	156.3	187.2	46.8	16.5	25.8	140.0	-3.00	0.0239
FXYD3	BC005238	190.4	225.5	115.0	100.2	54.5	112.4	-2.18	0.0264

Table III. Nucleotide sequences of the quantitative real-time PCR primers.

| Gene/Description	Microarray results	Primer sequences
A kinase (PRKA) anchor protein 8 (AKAPAL8)	Up-regulated	5'-GCAGGCAGGCAAGAAGAG-3' 5'-TGGCCATCTCGTCCTCAT-3'
Peptidase (mitochondrial processing) beta (PMPCB)	Up-regulated	5'-TTGAACGTGAGCGTGGAG-3' 5'-TTGTCCGTCCAAGTGCAG-3'
Phosphodiesterase 5A, cGMP-specific (PDE5A)	Up-regulated	5'-CAGCCGCCTCTTTGATGT-3' 5'-TTCAAGGGCTCACCAAGC-3'
Phosphatidylinositol transfer protein, alpha (PITPNA)	Down-regulated	5'-CGTCCTACCCCCATGTTG-3' 5'-ACTGGGCAGCGTCTGTTC-3'
Dihydrouridine synthase 2-like SMM1 homolog (DUS2L)	Down-regulated	5'-TGCTGGCCCTGGATTATG-3' 5'-ATCATCAGGGGCGACAAA-3'
RIC8A	Down-regulated	5'-AGGAAGACGCTGCCCTTT-3' 5'-GCAAGTTCCCCAGGAGGT-3'
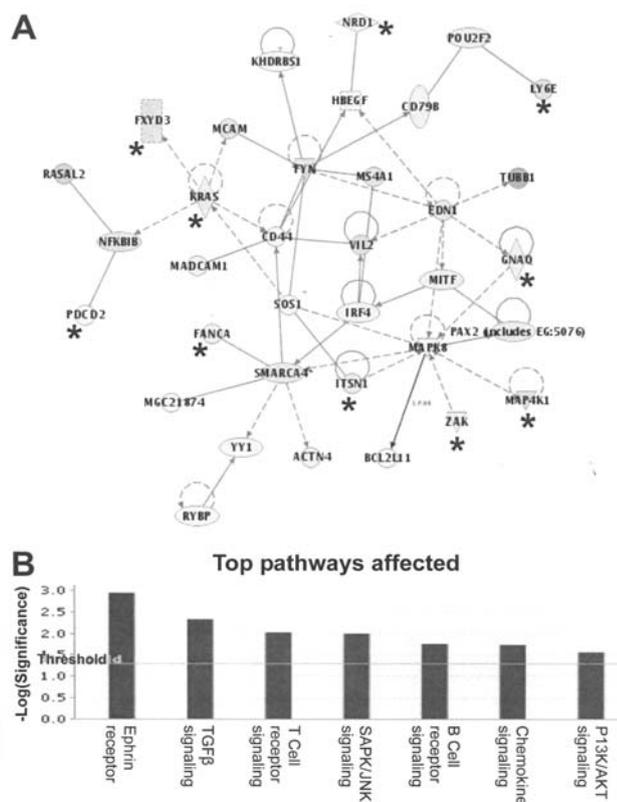
Figure 5. Ingenuity Pathways Analysis of the microarray data. (A) Ingenuity Network Analysis of Rg3 and S2h-regulated genes. Among the 409 significant genes, 35 genes are included this top network. Asterisk indicates down-regulated genes, whereas other shaded nodal points indicated up-regulated genes. (B) Ingenuity analysis of top pathways affected by both Rg3 and S2h. Y-axis is an inverse indication of p-value or significance. The Threshold line marks the p=0.05.

Figure 6. Quantitative real-time PCR analysis of three up-regulated genes, AKAPA8L, PMPCB and PDE5A. See text for details.

Top functions affected by Rg3 and S2h were the gene functions involved in cancer, cell morphology, gene expression, cell proliferation, and cell cycle, are among the most significantly affected functions by both Rg3 and S2h in human cancer line HCT-116 cells (data not shown).

Verification of ginsenoside Rg3- and S2h-regulated expression of candidate target genes using quantitative real-time PCR analysis. From the top 20 candidate gene lists shown in Tables I and II, we chose to determine if the expression of six target genes was regulated by Rg3 and S2h in HCT-116 cells. The six candidate targets include three up-regulated genes: A kinase (PRKA) anchor protein 8 (AKAPA8L), peptidase (mitochondrial processing) beta (PMPCB), and phospho-diesterase 5A cGMP-specific (PDE5A), and three down-regulated genes: phosphatidylinositol transfer protein, alpha (PITPNA), dihydrouridine synthase 2-like SMM1 homolog (DUS2L), and resistance to inhibitors of cholinesterase 8 homolog A (RIC8A). As shown in Fig. 6, the expression level of the 3 selected up-regulated genes, especially AKAPA8L, significantly increased upon Rg3 and S2h treatment (Fig. 6A). Conversely, the expression of the three down-regulated candidate genes decreased, more significantly at 48 h after treatment (Fig. 7). Thus, these results of Real-time PCR validated the findings from microarray analysis.
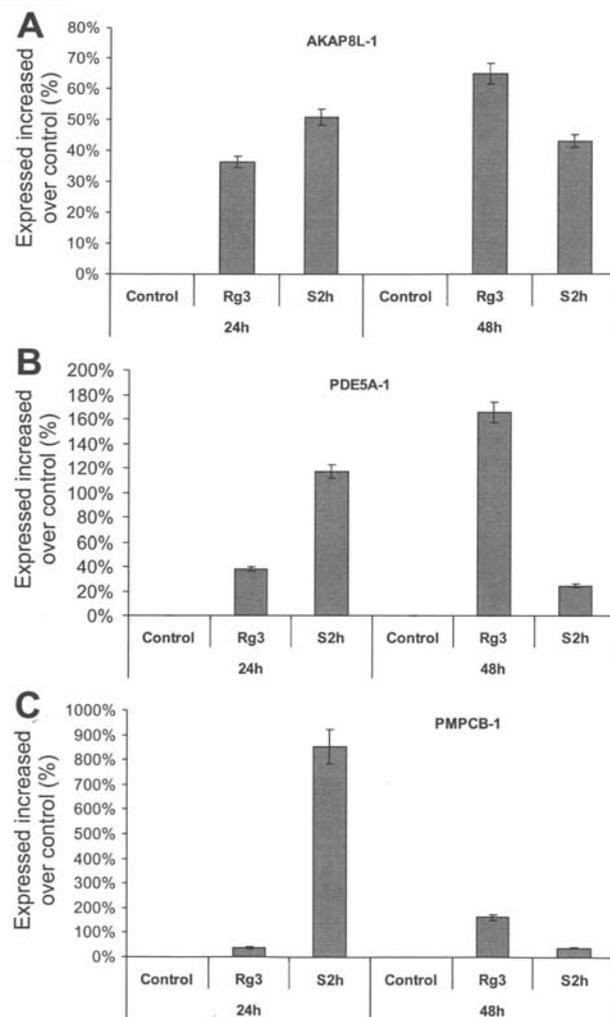
Discussion

Human colorectal cancer is a significant public health problem in the United States. This cancer is the second-leading cause of cancer-related deaths and the second most prevalent cancer worldwide (7,18). Although colon tumorigenesis may be one of the best-understood human solid tumors, the clinical management of advanced colon cancer remains highly challenging. The results of this study have demonstrated that the extracts from American ginseng exert significant anti-cancer activity in the colon cancer cells (11,12). It is believed that the anti-cancer activity results from the saponins, which are the main active constituents in American ginseng (e.g. ginsenosides Rg3). Additionally, we found that heating processing could increase the content of active constituent and enhance the anti-cancer activity, which may be the result of the relative augmentation of Rg3 constituency in steamed extracts.

The antiproliferative activities have demonstrated that both Rg3 and S2h exert effective anti-cancer activity. Thus, it is conceivable that the potentially important targets may be those candidate genes regulated by both Rg3 and S2h. In this study, we found that one of the most effected cell function
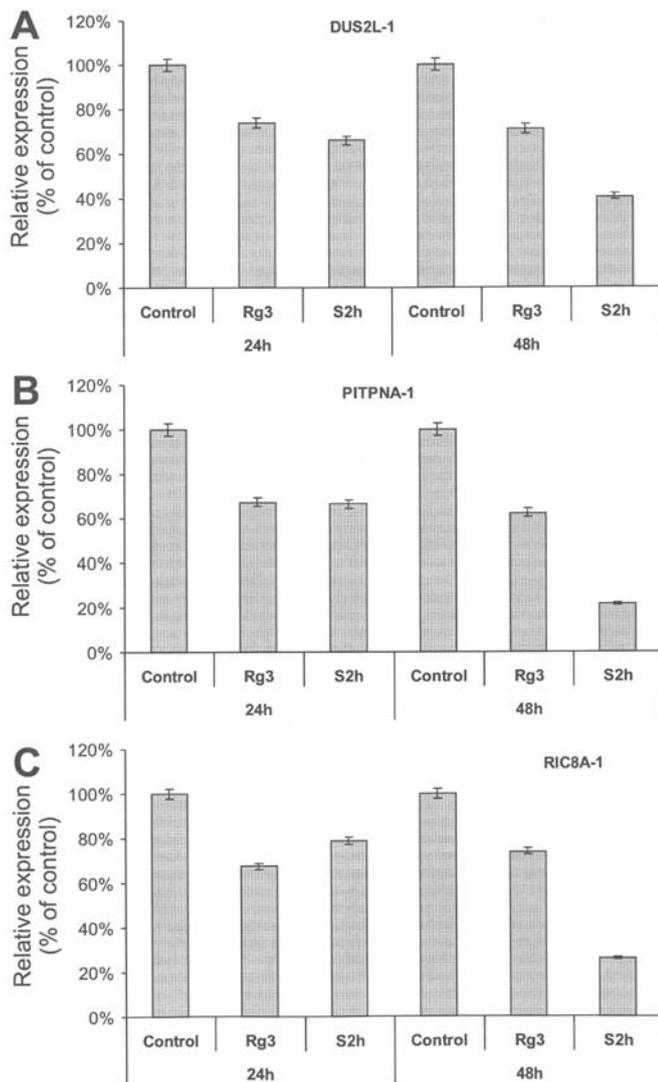
Figure 7. Quantitative real-time PCR analysis of three down-regulated genes, PITPNA, DUS2L and RIC8A. See text for details.

was tumorigenesis, which proved that the Rg3 and S2h are involved in the anti-cancer activity. The most effected pathway is the Ephrin receptor pathway. Eph receptors are the largest receptors in the tyrosine kinase family of transmembrane proteins, capable of recognizing signals from the cell environment and influencing cell-cell interaction and cell migration (19). When Ephrins bind to Eph receptors, the bi-directional signaling of the Eph/ephrin axis will be activated, which results in tumorigenesis as related to tumor growth and survival and is associated with angiogenesis and metastasis in many types of human cancer. Previously, the Eph/ephrin signaling as novel inhibition strategies of tumors has been investigated. Our results suggest that Rg3 and S2h may exert effective anti-cancer activity through the Eph/ephrin pathway.

In our study, we found that the expression levels were regulated by both Rg3 and S2h involved in different kinds of genes, such as genes related to signaling pathway, protein phosphorylation and cell proliferation. After searching the literature, the up-regulated gene AKAPA8L and down-regulated gene PITPNA are perhaps the most important target genes involved in anti-cancer activity of Rg3 and S2h. AKAPA8L belongs to a family of specific anchor proteins that target cAMP-dependent protein kinase A (PKA) at discrete subcellular locations (20,21). Besides interacting with PKA, AKAPs also bind other signaling molecules, mainly phosphatases and kinases, which regulate AKAP targeting and activate other signal transduction pathways (20,21). Thus, the AKAP transduceosome optimizes the amplitude and the signal/noise ratio of cAMP-PKA stimuli traveling from the membrane to the nucleus and other sub-cellular compartments. AKAP95, homologue of AKAPA8L, was identified to associate with the nuclear matrix and is thought to direct the distribution of PKAII during cell division (22,23). AKAP95 binds Eg7/condensin, a structural element that is implicated directly in chromosome condensation (24). Upon entry to mitosis, AKAP95 is released from the nuclear matrix and associates with chromatin. At mitosis, the disassembly of nuclear membranes and solubilization of nuclear matrix allow AKAP95 to interact with and recruit Eg7/condensins to chromatin. Association of AKAP95 with chromatin is required for correct chromosome condensation (24). AKAPA8L (HA95) contains two C2H2-type zinc finger motifs and also localizes to the nucleus (25). Like AKAP95, it co-localizes with condensed chromosomes at metaphase, suggesting that the two nuclear proteins have related cellular functions (25-28). A recent integrative system analysis of protein-protein interaction has mapped AKAPA8L as one of the components of TNFα/NFκB signaling pathway (29).

Another important gene, phosphatidylinositol transfer protein alpha (PITPNA) is a member of a diverse set of cytosolic phospholipid transfer proteins that are distinguished by their ability to transfer phospholipids between membranes *in vitro* (30). PITPNA participates in the supply of phos-phatidylinositol (PI) required for many cellular events including phospholipase C (PLC) beta and gamma signaling by G-protein-coupled receptors and receptor-tyrosine kinases, respectively. Protein kinase C has been known to modulate PLC signaling by G-protein-coupled receptors and receptor-tyrosine kinases. Thus, PITPNA is an important mediator of cell proliferation signaling pathways (30,31).

From the analysis of microarray hybridization, we found that the anti-cancer mechanism of Rg3 and S2h has many of the same characteristics, and alterations of gene expression level imply important information for exploring this mechanism. AKAPA8L and PITPNA genes suggest that American ginseng takes effect through regulating cell mitosis and some intracellular singling pathway. Our microarray analysis may lead to the identification of markers that predict the responsiveness of colon cancer cells to ginseng treatment. This important knowledge could further be used to develop ginseng derivatives as novel chemotherapeutic and/or chemo-preventive agents for human cancer.

Acknowledgements

References

1. Wang CZ, Mehendale SR and Yuan CS: Commonly used antioxidant botanicals: active constituents and their potential role in cardiovascular illness. Am J Chin Med 35: 543-558, 2007.
2. Yoo HH, Yokozawa T, Satoh A, et al: Effects of ginseng on the proliferation of human lung fibroblasts. Am J Chin Med 34: 137-146, 2006.
3. Wang CZ, Xie JT, Zhang B, et al: Chemopreventive effects of Panax notoginseng and its major constituents on SW480 human colorectal cancer cells. Int J Oncol 31: 1149-1156, 2007.
4. Kim HS, Lee EH, Ko SR, et al: Effects of ginsenosides Rg3 and Rh2 on the proliferation of prostate cancer cells. Arch Pharm Res 27: 429-435, 2004.
5. Koo HN, Jeong HJ, Choi IY, et al: Mountain grown ginseng induces apoptosis in HL-60 cells and its mechanism have little relation with TNF-alpha production. Am J Chin Med 35: 169-182, 2007.
6. Yun TK, Lee YS, Lee YH, et al: Anticarcinogenic effect of Panax ginseng C.A. Meyer and identification of active compounds. J Korean Med Sci 16 (Suppl.): S6-S18, 2001.
7. Corbit R, Ebbs S, King ML and Murphy LL: The influence of lead and arsenite on the inhibition of human breast cancer MCF-7 cell proliferation by American ginseng root (Panax quinquefolius L.). Life Sci 78: 1336-1340, 2006.
8. Mehendale S, Aung H, Wang A, et al: American ginseng berry extract and ginsenoside Re attenuate cisplatin-induced kaolin intake in rats. Cancer Chemother Pharmacol 56: 63-69, 2005.
9. Aung HH, Mehendale SR, Wang CZ, et al: Cisplatin's tumoricidal effect on human breast carcinoma MCF-7 cells was not attenuated by American ginseng. Cancer Chemother Pharmacol 59: 369-374, 2007.
10. Helms S: Cancer prevention and therapeutics: Panax ginseng. Altern Med Rev 9: 259-274, 2004.
11. Wang CZ, Aung HH, Ni M, et al: Red American ginseng: ginsenoside constituents and antiproliferative activities of heat-processed Panax quinquefolius roots. Planta Med 73: 669-674, 2007.
12. Wang CZ, Zhang B, Song WX, et al: Steamed American ginseng berry: ginsenoside analyses and anticancer activities. J Agric Food Chem 54: 9936-9942, 2006.
13. Peng Y, Kang Q, Luo Q, et al: Inhibitor of DNA binding/differentiation helix-loop-helix proteins mediate bone morphogenetic protein-induced osteoblast differentiation of mesenchymal stem cells. J Biol Chem 279: 32941-32949, 2004.
14. Park J, Rhee D and Lee Y: Biological activities and chemistry of saponins from Panax ginseng C. A. Meyer. Phytochem Rev 4: 159-175, 2005.
15. Wang CZ, McEntee E, Wicks S, et al: Phytochemical and analytical studies of Panax notoginseng (Burk.) F.H. Chen. J Nat Med 60: 97-106, 2006.
16. Wang CZ, Wu JA, McEntee E and Yuan CS: Saponins composition in American ginseng leaf and berry assayed by high-performance liquid chromatography. J Agric Food Chem 54: 2261-2266, 2006.
17. Li C and Wong WH: Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. Proc Natl Acad Sci USA 98: 31-36, 2001.
18. Jemal A, Tiwari RC, Murray T, et al: Cancer statistics, 2004. CA Cancer J Clin 54: 8-29, 2004.
19. Surawska H, Ma PC and Salgia R: The role of ephrins and Eph receptors in cancer. Cytokine Growth Factor Rev 15: 419-433, 2004.
20. Feliciello A, Gottesman ME and Avvedimento EV: The biological functions of A-kinase anchor proteins. J Mol Biol 308: 99-114, 2001.
21. Carnegie GK and Scott JD: A-kinase anchoring proteins and neuronal signaling mechanisms. Genes Dev 17: 1557-1568, 2003.
22. Coghlan VM, Langeberg LK, Fernandez A, et al: Cloning and characterization of AKAP 95, a nuclear protein that associates with the regulatory subunit of type II cAMP-dependent protein kinase. J Biol Chem 269: 7658-7665, 1994.
23. Collas P, Le Guellec K and Tasken K: The A-kinase-anchoring protein AKAP95 is a multivalent protein with a key role in chromatin condensation at mitosis. J Cell Biol 147: 1167-1180, 1999.
24. Steen RL, Cubizolles F, Le Guellec K and Collas P: A kinase-anchoring protein (AKAP)95 recruits human chromosome-associated protein (hCAP)-D2/Eg7 for chromosome condensation in mitotic extract. J Cell Biol 149: 531-536, 2000.
25. Orstavik S, Eide T, Collas P, et al: Identification, cloning and characterization of a novel nuclear protein, HA95, homologous to A-kinase anchoring protein 95. Biol Cell 92: 27-37, 2000.
26. Martins SB, Eide T, Steen RL, et al: HA95 is a protein of the chromatin and nuclear matrix regulating nuclear envelope dynamics. J Cell Sci 113: 3703-3713, 2000.
27. Martins S, Eikvar S, Furukawa K and Collas P: HA95 and LAP2 beta mediate a novel chromatin-nuclear envelope interaction implicated in initiation of DNA replication. J Cell Biol 160: 177-188, 2003.
28. Martins SB, Marstad A and Collas P: In vitro modulation of the interaction between HA95 and LAP2beta by cAMP signaling. Biochemistry 42: 10456-10461, 2003.
29. Bouwmeester T, Bauch A, Ruffner H, et al: A physical and functional map of the human TNF-alpha/NF-kappa B signal transduction pathway. Nat Cell Biol 6: 97-105, 2004.
30. Wirtz KW: Phospholipid transfer proteins in perspective. FEBS Lett 580: 5436-5441, 2006.
31. Phillips SE, Vincent P, Rizzieri KE, et al: The diverse biological functions of phosphatidylinositol transfer proteins in eukaryotes. Crit Rev Biochem Mol Biol 41: 21-49, 2006.