Proteomic characterization of the cellular response to chemopreventive triterpenoid astragaloside IV in human hepatocellular carcinoma cell line HepG2

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Abstract. Triterpenoids are implicated in the chemoprevention of various cancers. Current challenge is to define the molecular mechanism underlying the chemopreventive activity of triterpenoids. This study was designed to characterize the intracellular proteins regulated by astragaloside IV, the major active triterpenoid in Radix Astragali. Upon the treatment with astragaloside IV, human hepatocellular carcinoma HepG2 cells were evaluated for the colonogenic survival and anchorage-independent growth. The cellular proteins of treated and untreated cells were resolved by 2-D polyacrylamide gel electrophoresis. The protein spots mostly altered by drug treatment were identified by mass spectrometry and subsequently verified by Western blotting using specific antibodies and RT-PCR technique using specific DNA primers. We found that astragaloside IV attenuated the colonogenic survival and anchorage-independent growth of cancer cells. Based on the proteomic profiles, top 14 upregulated and 13 down-regulated protein spots were subjected to mass spectrometric analysis. As an example, Vav3.1 belongs to the oncogene Vav family, which is implicated in tumorigenesis. Vav3.1 expression was down-regulated by astragaloside IV in a dose- and time-dependent manner. Down-regulation of Vav3.1 was highly correlated with the suppression of cell malignant transform. Thus, astragaloside IV may elicit anticancer activity via down-regulating the expression of oncogenes such as Vav3.1.

Introduction

Tumorigenesis is a complex pathological process involving three distinct stages of initiation, promotion and progression (1). Although several etiologies exist, aberrant activation of various oncogenes is a key intracellular event in the process of tumorigenesis (2,3). Activated oncogenes disrupt the cellular signals that govern the cell proliferation, differentiation and apoptosis (4,5). Some oncogenes may intervene with the intercellular signal transduction normally mediated by growth factors, neurotrophic factors and cytokines (6-8). For example, a family of three 21-kDa Ras proteins, namely, H-Ras, N-Ras and K-Ras, are often activated in the process of cancer development (7,9-11). Notably, the activity of Ras proteins is highly dependent on the replacement of bound guanosine diphosphate (GDP) with guanosine triphosphate (GTP), an enzymatic reaction catalyzed by a large group of guanine nucleotide exchange factors (GEFs) including SOS-1/2, CDC25Mm and Vav proteins (7,10-13). There is compelling evidence supporting that GEFs such as Vav proteins (Vav1, Vav2 and Vav3) are hyperactive in various cancers (14-17). Equally importantly, RNAi-mediated knock-down of Vav3 expression resulted in significant regression of tumor growth (15,17). Recent mechanistic studies demonstrated that Vav proteins activate a panel of small GTPases such as Rho, Rac and Cdc42 (18-21). Active Rho, Rac and Cdc42 subsequently stimulate the formation of focal adhesion complexes involving integrins, paxillin, vinculin and focal adhesion kinase (FAK), and induce membrane ruffling and filopodia formation (14,20,22). Rearrangement of cytoskeleton structure is a key event in cell malignant transformation. In addition, Vav proteins directly interact with Grb2, Shc and the p85 subunit of PI3K and subsequently modulate receptor tyrosine kinase signaling pathways, leading to the activation of Akt, MAPK and Stat3 (23-27). These results highlight the possibility that GEFs including Vav proteins may be therapeutic targets in inhibiting cell oncogenic transformation (23,28-30).

Radix Astragali is a dietary supplement widely used in traditional Chinese medicine to prevent and treat various...
cancers (31). The molecular basis of the anticancer activity of Radix Astragali extract has not been fully explored. Triterpenoid astragalosides and polysaccharides were suggested to be active against cancers (32,33). Recent studies showed that herbal polysaccharides modulate human immune system in a non-specific manner (34,35). The total astragaloside extract of Radix Astragali inhibited the growth of human colon cancer cells in cell culture system and in a xenograft cancer model (33). Astragaloside IV represents a group of cycloartane-type triterpenoid glycosides and is used as a marker compound in Radix Astragali (Fig. 1). Several cycloartane-type triterpenoid glycosides are found to be chemopreventive and structurally related ginsenoside Rg3 is clinically applied to treat cancers (36). These results motivated us to investigate the anticancer activity of astragalosides with the hope of developing a new cancer therapy.

In the present study, we investigated the effects of astragaloside IV on the colony formation of tumor cells. To characterize the cellular targets for astragaloside IV, we compared the proteomic profiles of human liver cancer cells treated with astragaloside IV or vehicle, respectively. Owing to its potential in tumorigenesis, the regulation of oncogene Vav3.1 by astragaloside IV was further investigated by RT-PCR technique and Western blotting.

Materials and methods

Cell culture and reagents. Human hepatocellular carcinoma cell line HepG2 was obtained from American Type Culture Collection (Rockville, MD, USA) and cultured in Eagle’s minimum essential medium (MEM) containing 10% fetal bovine serum at 37°C in the incubator equilibrated with 5%CO2 for 24 h. The cells were cultured at 37°C in the incubator equilibrated with 5%CO2 for 24 h. At the end of the treatment, the cells were harvested and washed twice with ice-cold PBS. The cells were cultured at 37°C in the incubator equilibrated with 5%CO2 for 14 days. The clones with a diameter <25 μm were observed and counted under an inverted image difference microscope. The clonogenic fraction was calculated by the following equation: colony formation rate (%) = (clone number/cells seeded) x 100%. This experiment was performed with three biological replicates.

Cell proliferation assay. Following the 3-day treatment with astragaloside IV (150 μg/ml) or vehicle alone consecutively for 3 days, ASIV-treated and control HepG2 cells were examined for the anchorage-independent growth in agarose. The cells were trypsinized and washed twice with serum-free medium, and centrifuged for 5 min at 1200 rpm, and re-suspended at the density of 400 cells/ml in the complete growth medium supplemented with 10 μg/ml of astragaloside IV or vehicle alone. The bottom agarose was prepared by mixing equal volume of sterilized 1.2% agar with 2X MEM supplemented with 20% fetal bovine serum at 42°C, applied into a 6-well cell culture plate by 3 ml per well and cooled down to solid. The remaining agarose was mixed equivalently with the cell suspension containing 150 μg/ml of astragaloside IV or vehicle alone. The mixture was immediately poured into the cell culture plate at 2 ml per well, 400 cells per well. The cells were cultured at 37°C in the incubator equilibrated with 5%CO2 for 14 days. The clones with a diameter >50 μm were observed and counted under an inverted image difference microscope. The clonogenic survival was calculated by the following equation: clonogenic survival (%) = (clone number/cells seeded) x 100%. This experiment was performed with three biological replicates.

Two-dimensional (2-D) gel electrophoresis. HepG2 cells were treated with astragaloside IV (200 μg/ml) or vehicle alone at 37°C for 24 h. At the end of the treatment, the cells were harvested and washed twice with ice-cold PBS. The cells were pelleted and lysed with a 40 mM Tris buffer (pH 7.4) containing 8 M urea, 4% CHAPS, 2% ampholine, and 65 mM dithiothreitol (DTT). The cellular proteins were recovered following centrifugation at 20,000 x g for 60 min at 4°C, and protein concentration was measured using the Bradford assay (37). Proteins were separated by 2-D electrophoresis essentially according to the manufacturer’s instructions (Amersham Biosciences, Uppsala, Sweden). For isoelectric focusing (IEF), 300 μg of cellular proteins were mixed with a rehydration solution containing 2 M thiourea, 7 M urea, 2% CHAPS, 0.5% immobiolized-pH-gradient (IPG) buffer (pH 4.0-7.0) and 10 mM DTT. The IEF was subsequently carried out in 24 cm Immobiline Drystrips (pH 4.0-7.0) using an ETTAN IPGphor 3 apparatus (GE Healthcare). The focusing was achieved sequentially at 50 V for 24 h, 500 V for 1 h, 1,000 V for 1 h, and then held at 8,000 V until current dropped <25 μA and for a total of >100,000 V.h. When the IEF was completed, the individual strips were equilibrated in 50 mM Tris-HCl buffer (pH 8.8) containing 6 M urea, 30% glycerol, 2% SDS, 1% DTT and a trace of bromophenol blue. The free thiol groups were inactivated with 2.5% iodoacetamide in the same buffer for another 20 min. After the IPG strips were
mounted on the top of the gels, proteins were resolved in 12.5% SDS gels using the Ettan Dalt six electrophoresis unit (GE Healthcare). SDS-PAGE was run at 20 W/gel for 4 h until the bromophenol blue front reached the bottom of the gel.

**Protein imaging and analysis.** The proteins resolved in the gel were visualized by a standard silver staining procedure described in the manufacturer's instructions (Amersham Biosciences). The stained gels were scanned using ImagerScanner with LabScan software version 5.0 (GE Healthcare) and analyzed using ImageMaster Platinum software version 5.0 (GE Healthcare). Image analysis included spot detection, spot editing, background subtraction, and spot matching. The resulting data were exported to Microsoft Excel for comparison.

**In-gel proteolytic digestion.** An automated spot-picker was used to recover the spots of interest from the 2D gels to corresponding siliconized Eppendorf tubes. The silver stain was removed by a freshly prepared destaining solution [30 mM potassium hexacyanoferrate: 100 mM sodium thiosulfate, 1:1 (v/v)]. After a couple of rinses with water, the gel pieces were incubated in 100 mM ammonium bicarbonate for 5 min, and then dehydrated with 100% acetonitrile. The residual solvents were removed in a vacuum centrifuge. Proteins were digested by 20 μg/ml proteomics sequencing grade trypsin (Promega) in the ice-cold buffer containing 40 mM ammonium bicarbonate, 9% acetonitrile. The peptides were extracted twice by adding 5% formic acid, 50% acetonitrile for 15 min at room temperature. The resulting solutions were dried in a vacuum centrifuge.

**Peptide identification by MALDI-TOF mass spectrometry.** The peptide residues were dissolved in 10 μl of 0.1% formic acid. Peptide mixtures (1 μl) were then mixed with an equal volume of 10 mg/ml α-cyano-4-hydroxycinnamic acid (Sigma) saturated with 50% acetonitrile/0.1% formic acid and spotted onto a MALDI target plate. The peptide spots were analyzed with an ABI 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA). The MS setting was positive reflector mode. Laser shots of 200 per spectrum were used to acquire spectra with a mass range of 900-4,000 Da with a focus mass of 2,000 Da. The MS/MS setting was 2 kV positive/CID on and 5 monoisotopic precursors selected (S/N>200). The calibration was performed using the Calibration mixture 1 of 4,700 Proteomics Analyzer calibration mixture (Applied Biosystems). The spectra were calibrated externally using P14R and insulin chain B oxidized from bovine pancreas (Sigma). Autolytic peaks of trypsin served as internal standards for mass calibration. A combined database search of PMF and MS/MS data was performed using GPS Explorer™ Workstation (Applied Biosystems), and the NCBInr database was selected in the MASCOT search engine (www.matrix-science.com). Search parameters included Homo sapiens, trypsin digest (allowed up to 1 missed cleavage), cysteines modified by carbamidomethylation, methionine modified by oxidation, and the mass tolerance was set at 75 ppm. Probability scores greater than 63 were defined as significant (p<0.05). In cases of low score matching, duplicate runs were made to ensure the accuracy of analysis. The protein spots were annotated by searching Gene Ontology (http://www.geneontology.org/).

**Western blot analysis.** Total cell lysates containing 30 μg of proteins were resolved by 12% SDS-PAGE, and were subsequently transferred onto PVDF transfer membrane (GE Healthcare Life Sciences). Membranes were blocked with 5% non-fat milk in TBST (50 mM Tris-Cl, pH 7.6, 150 mM NaCl, and 0.1% Tween-20) at 4°C overnight. The membranes were then probed with the antibodies specific for the indicated proteins (1:1,000 dilution) at room temperature for 1 h. The bound antibodies were detected by horseradish peroxidase (HRP)-conjugated secondary antibody (1:1,500 dilution) at room temperature for 1 h and then visualized by enhanced chemiluminescence (ECL) reaction reagents (GE Healthcare Life Sciences). Protein loading was normalized by re-probing the same membranes with β-actin specific antibodies.

**RT-PCR detection.** Following the treatment with astragaloside IV or vehicle alone, total RNA was isolated using RNeasy kit (Qiagen, USA) and subsequently converted into cDNA templates by reverse transcription using SuperScript II RT kit and random hexamer primers (Invitrogen, USA). The intracellular mRNAs encoding VAV3.1 (NM_0010797874) and β-actin (NM_001101) were determined by PCR technique with AccuPrime™ Taq DNA polymerase (Invitrogen) and specific primers: VAV3.1 sense: 5'-CCAATTTTATACATTTCTTCCAGA-3', VAV3.1 antisense: 5'-TTTACATCTCCTTCCACATATG-3'; β-actin sense: 5'-GGCACCACACCTTCTCCTTTCAGA-3', β-actin antisense: 5'-GGAGTTGAAGGTTGGAATTTTGTTGGAAGGTA-3'. The resultant PCR products were analyzed by 1.2% agarose gel electrophoresis.

**Statistical analysis.** Statistical analysis was performed by Microsoft Excel 2003. All means were calculated from three experiment replicates, and the error bars represent standard error of mean (SEM). All data are expressed as means ± SEM. Statistical significance was determined by paired and two-tailed Student's t-test. A p<0.05 was considered statistically significant.

**Results**

**Inhibition of tumorigenesis by astragaloside IV.** To achieve a pharmacologically altered cell phenotype, HepG2 cells were treated with astragaloside IV for three consecutive days. Astragaloside IV-treated and untreated HepG2 cells were examined for the proliferation, focus formation and anchorage-independent growth in soft agar. Under conventional cell culture conditions, astragaloside IV did not show any alteration in the proliferation of HepG2 cells (data not shown). This result supports the clinical use of astragaloside IV-containing Radix Astragali in a large quantity without causing evident cytotoxicity (38). When the cells were cultured in the growth medium supplemented with 5% fetal bovine serum, however, astragaloside IV-treated cells showed a dramatic decrease in the colonogenic survival in the culture dishes (Fig. 2A). Astragaloside IV also exhibited an inhibitory effect on the anchorage-independent growth of HepG2 cells in the soft agar colony assay.
agah (Fig. 1B). Importantly, transformed cells are able to survive and grow in an anchorage-independent manner (39). Thus, our results suggest for the first time that astragaloside IV is active in inhibiting the colony formation of HepG2 cells as a surrogate of liver cancer.

Identification of the proteins mostly affected by astragaloside IV. To elucidate the molecular mechanisms underlying the inhibition of astragaloside IV on tumorigenesis, we examined the effect of astragaloside IV on the protein expression in human cancer cells. The cellular proteins obtained from astragaloside IV-treated or untreated HepG2 cells were resolved by 2D gel electrophoresis (Fig. 3). After spot detection, spot editing and spot matching, the protein map of astragaloside IV-treated cells was quantitatively compared with that of untreated cells using ImageMaster Platinum software version 5.0 (GE Healthcare). The protein spots were classified into two different groups: up-regulated and down-regulated, respectively (Table I). According to the rank of the fold change, 14 upregulated protein spots were picked up from the astragaloside IV-treated samples, whereas 13 downregulated protein spots were obtained from the untreated samples. The selected protein spots were subjected to in-gel digestion and subsequent MALDI-TOF mass spectrometry.
The protein identities corresponding to individual spots were annotated using Mascot software (http://www.matrixscience.com) against public protein database (NCBInr). As shown in Table I, the cellular responses to astragaloside IV treatment can be classified into three functional categories: up-regulated group, up/down-regulated group and down-regulated group, respectively. Firstly, astragaloside IV appeared to enhance the cellular response to stress stimuli. Several stress responsive proteins such as glucose-regulated protein BiP/GRP78 (NP_005338), heat shock 70 kDa protein 2 (NP_068814), HSPA1A protein (NP_005336) and HSPA8 protein (NP_006588) and chaperon containing TCP1, subunit 8 (ı) (NP_006576), belong to the up-regulated group. It is interesting that several astragaloside IV-affected proteins are involved in protein synthesis, especially the initiation stage. Eukaryotic translation initiation factor 3S2b (NP_003748) and eukaryotic translation initiation factor 4A1 (NP_001407) are also upregulated. Moreover, proliferation associated 2G4 protein (AAH32111) is an RNA-binding protein that is involved in the regulation of cell growth (40). Our results also showed that the expression of proliferation-associated proteins was enhanced by astragaloside IV. Secondly, the proteins involved in cytoskeleton organization and protein degradation were either positively or negatively regulated by astragaloside IV. For example, structural proteins including desmin (NP_001918), keratin 5 (NP_000415), tubulin α-1B (P68363), β-actin (NP_001092) and cytokeratin (NP_002264) were upregulated up to 5.8-fold, whereas a few others such as keratin 86 (NP_002275), plectin (NP_000436), cytokeratin type II (NP_004684) and outer dense fiber of sperm tails 2-like...
isoform b (NP_001007023) were downregulated by a range from -2.3 to -13.2. On the other hand, proteasome 26S ATPase subunit 3 (NP_002795) and ubiquitin-conjugating enzyme E2N-like (NP_001013007) were upregulated by 4.7- and -2.6-fold, respectively. Thirdly, the regulatory proteins involved in cell signaling, differentiation and transcription were downregulated up to -12.1-fold. These proteins include stomatin (EPB72)-like 2 (NP_038470), M6-kinase 3 (NP_060142), regulator of G protein signaling 9 (NP_003826), stathmin-1 (NP_981946) and zinc finger protein ZNF45 (NP_003416) and Vav3 protein. However, the Vav3-specific antibody could not distinguish two forms from each other (Fig. 4A). Based on the location of spot, the protein was tentatively assigned as Vav3.1 (NP_001073343). Vav3 is highly homologous to Vav1 and Vav2 both in mRNA sequence and amino acid sequence, whereas Vav3.1 is only composed of 287 amino acid residues, corresponding to the peptidase inhibitor B7 (NP_001035237) and peroxiredoxin 6 (NP_004896) are directly involved in cell signal transduction, the cellular levels of these two proteins were also decreased respectively by 3.3- and 2.3-fold in response to astragaloside IV. Collectively, our results suggest that astragaloside IV may affect cell proliferation and differentiation through decreasing the expression of some important regulatory proteins.

### Verification of Vav3.1 expression
The protein spots corresponding to Vav3.1 were carefully compared by amplifying the protein image (Fig. 4B). To verify the suppression of Vav3.1 by astragaloside IV, the cellular proteins obtained from astragaloside IV-treated and untreated cells were resolved by 12% SDS-PAGE. The proteins in the gel were transferred...
onto PVDF membrane, the membrane was subsequently probed by specific antibodies against Vav3 and β-actin, respectively (Fig. 4C). Consistent with the results of previous 2D-electrophoresis analysis (Fig. 3), the antibody detected a peptide band, which matched the protein Vav3.1 (NP_001073343) in size (Fig. 4C). The signal intensity of Vav3.1 spot was decreased by 1.7-fold in astragaloside IV-treated sample compared with that of control sample. In order to overcome the difficulty of distinguishing Vav3 isoforms by using Vav3-specific polyclonal antibodies, oligonucleotide primers were specifically designed for the major transcript vav3.1 (NM_001079874) encoding Vav3.1 isoform. The PCR products are shown in Fig. 4C, in which Vav3.1 mRNA was clearly down-regulated by astragaloside IV. Such a result was highly consistent with the previous DNA microarray analysis (38). Thus, suppression of oncogenic Vav3.1 expression may represent a key mechanism underlying the action of astragaloside IV.

**Regulation of Vav3.1 expression by astragaloside IV.** Using a specific antibody, a panel of human cell lines were screened for Vav3.1 expression. As shown in Fig. 5, Vav3.1 was detected in HepG2, T47D, MB-AMD-231 PC-3 and HEK 293T cells at various levels, whereas no signal was detectable in MCF-7 and LNCaP cells, suggesting that Vav3.1 expression is cell-type dependent. Among three breast cancer cell lines, MB-AMD-132 and T47D are more invasive than MCF-7 (43). Similarly, PC-3 is a highly aggressive and androgen-independent prostate cancer cell line compared with LNCaP cells (44). Conceivably, Vav3.1 expression is positively correlated with the tumor aggressiveness. To understand the pharmacological regulation of Vav3.1 expression, HepG2 cells were used as the cellular detector and treated with astragaloside IV at the indicated doses of 0, 50, 100, 150 and 200 μg/ml. As shown in Fig. 6A, Vav3.1 expression was suppressed by astragaloside IV in a dose-dependent manner. It is evident that Vav3.1 expression was strongly inhibited by astragaloside IV at the dose of 150 μg/ml and higher (Fig. 6B and C). We also observed a clear increase in the intracellular level of Vav3.1 with the prolonged cell culture under the normal conditions. These results stimulate us to postulate that suppression of Vav3.1 expression may be a key mechanism supporting the inhibition of tumorigenesis by astragaloside IV.
subsequently transferred onto PVDF membrane. The membrane was probed with specific rabbit anti-Vav3 and anti-ß-actin antibodies. The bound antibodies were detected by goat anti-rabbit IgG-HRP conjugates and visualized by ECL kit. (B) Time course. The cells were cultured in the specific growth medium and harvested when the cell density reached 70-80% confluence. The cell lysates were resolved by 12% SDS-PAGE and subsequently transferred onto PVDF membrane. The membrane was probed with specific rabbit anti-Vav3 and anti-ß-actin antibodies. The bound antibodies were detected by goat anti-rabbit IgG-HRP conjugates and visualized by ECL kit. (C) Suppression of Vav3.1 expression in HepG2 cells. The proteins mostly affected by astragaloside IV were identified by MALDI/MS technology and their biological functions were annotated against public protein databases. According to our proteomic analysis, astragaloside IV affected the expression of a panel of proteins, especially the proteins involved in the stress response, protein biosynthesis and cell signaling pathways. Their associations with oncogenic transformation are discussed.

First of all, most of the significantly regulated proteins by astragaloside IV are involved in the cellular response to stress. Stress proteins include glucose-regulated protein BiP/GRP78 (NP_005338), heat shock 70 kDa protein 2 (NP_068814), HSPA1A protein (NP_005336) and HSPA8 protein (NP_006588). Chaperon containing TCP1, subunit 8 (0) (NP_006576) is not fully characterized, but likely to be a stress protein. When the cells are challenged by various stresses, heat shock proteins are coordinately upregulated whereas many other genes that were active before the metabolic insult are downregulated (46,47). Heat shock proteins and other stress proteins normally function as molecular chaperones. These molecular chaperones interact with a panel proteins involved in several mechanisms, leading to the inhibition of the cellular inflammatory responses (47,48). It is not known if astragaloside IV treatment imposes a kind of stress to the cells, the increased gene expression of stress proteins is critical to limit cell damage and facilitate the continued cell survival.

Secondly, our results also suggested that translation may be a key biological process targeted by astragaloside IV. Translation initiation enables the generation of functional gene products and thus plays a key role in the overall process of gene expression. In translation, messenger RNA (mRNA) is decoded to produce a specific polypeptide or protein according to the rules specified by the genetic code (49). Translation occurs in the cytoplasm where the ribosomes are located. Protein synthesis proceeds in four phases: activation, initiation, elongation and termination (49). Eukaryotic translation initiation factors (IF) and other proteins facilitate the binding of the small subunit of the ribosome to the 5’ end of mRNA, a key event to initiate the synthesis of a protein. Translation initiation factor eIF2 is essential for the initiation of protein synthesis because it protects formyl-methionyl-tRNA from spontaneous hydrolysis as well as promotes the binding of Met-tRNA complex to the 30S ribosomal subunits. In addition, the factor eIF2 takes part in the hydrolysis of GTP during the formation of the 70S ribosomal complex. Translation initiation factor eIF3 is the largest mammalian translation initiation factor consisting of at least eight subunits with the mass ranging from 35 to 170 kDa (50). Factor eIF3 forms a complex with the 40S ribosome in an early step of translation initiation and promotes the pairing of initiation Met-tRNA and mRNA. Eukaryotic translation initiation factor 3 subunit 28 is a 36-kDa protein containing WD40 repeats, eIF3 subunit 28 was found to be identical to TRIP-1, a phosphorylation substrate of the TGF-ß type II receptor (50,51). Translation initiation has been a major target for drug development (52). A variety of antibiotics such as anisomycin, cycloheximide, chloramphenicol, tetra-cycline, streptomycin, erythromycin, puromycin have been used to disable or inhibit translation in protein biosynthesis (52).

Therefore, the biological importance of the regulation of translation initiation factors by astragaloside IV should be further addressed.

Discussion

A wide range of phytochemicals are effective to prevent and inhibit tumor initiation, promotion and progression (45). Radix Astragali is an important medicinal herb widely used in traditional Chinese medicine to treat various cancers (31). We recently observed that the major active compound astragaloside IV in the herb attenuated the ability of HepG2 cells to form cell foci and to grow in the anchorage-independent manner, suggesting a novel anticancer mechanism for this group of triterpenoids. The objective of this study was to further investigate the effects of astragaloside IV on protein expression in HepG2 cells. The proteins mostly affected by astragaloside IV were identified by MALDI/MS technology and their biological functions were annotated against public protein databases. According to our proteomic analysis, astragaloside IV affected the expression of a panel of proteins, especially the proteins involved in the stress response, protein biosynthesis and cell signaling pathways. Their associations with oncogenic transformation are discussed.

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Therefore, the biological importance of the regulation of translation initiation factors by astragaloside IV should be further addressed.
Thirdly, our results showed that astragaloside IV altered the cellular level of several proteins involved in the cell signaling (Table I). M6-kinase 3 (NP_060142) is referred to as transient receptor potential cation channel (TRPC), subfamily M, member 7, and functions as both an ion channel and a kinase (53). As a channel, M6-kinase capacitates calcium entry into the cell. As a kinase, it catalyzes the phosphorylation of itself and other substrates. Importantly, its kinase activity is necessary for channel function. Intracellular ATP stimulates its kinase activity but PIP(2) hydrolysis causes the inactivation of the channel (54,55). Several studies demonstrated a role for this group of proteins in cell survival and proliferation (56,57). Regulator of G protein signaling 9 (RGS9) (NP_003826) is a member of the signaling protein RGS family that promote the GTPase activity of the G-protein coupled receptor proteins (58). Expression of mutated RGS9 was found in patients with bradyopsia, a disease with slow photoreceptor deactivation (59). Stathmin-1 (NP_981946) is a ubiquitously expressed signaling protein RGS9 is a member of the signaling protein RGS family that promote the GTPase activity of the G-protein coupled receptor proteins (58).

Conceivably, Vav3.1 protein may substitute for Vav3 in functions mediated by this domain and compete with Vav3 in functions mediated by N-terminal motifs. Nevertheless, our results suggest that pharmacological suppression of Vav3.1 may lead to the inhibition of tumorigenesis.

To explore the potential of Vav3.1 as a molecular target in cancer therapy, the regulation of Vav3.1 expression should be characterized at transcriptional and post-transcriptional levels. Growth factors TGF-β and EGF were found to down-regulate the expression of Vav3.1 transcript in HaCaT keratinocytes, indicating a growth factor regulated mechanism (41). Here we showed for the first time that astragaloside IV down-regulated both Vav3.1 mRNA and protein in a dose- and time-dependent manner. Thus, our results suggest a novel mechanism to support pharmacological intervention of cell malignant transformation by traditional herbal medicines.

In conclusion, we demonstrated that astragaloside IV altered the expression levels of several biologically important proteins in human HepG2 cells. Suppression of Vav3.1 expression appeared to be highly correlated with the inhibition of the cellular malignant transformation by astragaloside IV. Thus, when the role of Vav3.1 in the inhibitory effect of astragaloside IV on tumor formation is further clarified, it should be possible to develop a novel astragaloside IV-based anticancer therapy.

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