Proteomics Analysis of Kojic Acid Treated A375 Human Malignant Melanoma Cells

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Received December 21, 2007

Although the toxicogenomics of kojic acid treated A375 human malignant melanoma cells has been elucidated, the proteomics of cellular response is still poorly understood. We performed proteomic analysis to investigate the anticancer effect of kojic acid on protein expression profile in A375 cells. A375 cells were treated with kojic acid at 8 $\mu\text{g}/\text{mL}$ for 24, 48, and 72 h. With the use of 2-D PAGE and MALDI-Q-TOF MS and MS/MS analyses, proteomic profiles of A375 cells between control and kojic acid treatment were compared, and 30 differentially expressed proteins, containing 2 up-regulated proteins and 28 down-regulated proteins, were identified. Among these proteins, 17 isoforms of 5 identical proteins were observed and 11 chaperone proteins showed the high proportion of protein spots with 36.7% of total proteins. Bioinformatic tools were used to search for protein function and prediction of protein interaction. Sixteen differentially expressed proteins exhibited interaction network linked to the downstream regulations of p53 tumor suppressor and cell apoptosis, which may lead to suppress the melanogenesis and tumorigenesis of kojic acid treated A375 cells. In addition, GRP75, VIME and 2AAA were validated by Western blot analysis, whereas GRP75, 2AAA, HS90B, ENPL and KPYM were validated by RT-PCR. Therefore, these proteins play the important roles in cancer progression and may be potential biomarkers that are useful for diagnostic and therapeutic applications of malignant melanoma cancer.

Keywords: kojic acid • A375 cells • malignant melanoma • proteomics • cancer

Introduction

Kojic acid (5-hydroxy-2-hydroxymethyl-1,4-pyrone) is a secondary metabolic product and widely used as a food additive for preventing enzymatic discoloration of vegetable, crabs and shrimps^{1,2} and as a skin lightening or bleaching agent in cosmetic preparations.^{3,4} Although the reported safety aspects of kojic acid used in food additive and cosmetics have shown no human health risk,³ there are some reports which have shown the evaluation of the tumorigenic potential of kojic acid and its genotoxic effects in several *in vitro* tests, including mutation in *Salmonella typhimurium* strains TA98, TA100 and

TA1535, and sister chromatid exchanges and chromosome aberrations test in Chinese hamster ovary (CHO) cells.⁵⁻⁷ Chronic oral administration of kojic acid has been reported to produce thyroid adenomas in mice and rats.^{8,9} In contrast to *in vivo* studies, genotoxic activity of kojic acid was negative. The genotoxicity and carcinogenicity in rat liver and the photogenotoxicity and carcinogenicity in mouse skin were not found.¹⁰ After kojic acid administration in drinking water, the tumor initiation activity was not found in the rat thyroid.¹¹ As a skin lightening agent, the frequency of sensitization of skin care containing kojic acid was studied in female patients with suspected cosmetic-related contact dermatitis. It is considered to have high sensitizing potential, as a comparatively high frequency of contact sensitivity was observed.¹² The topical use of kojic acid as a skin lightening agent also resulted in minimal exposure but it has negligible risk of genotoxicity or toxicity in humans.¹³ Although the tumorigenic potential and some genotoxic effect of kojic acid on human skin cell lines has been widely studied, the effect of kojic acid on protein expression level that mostly possesses many biological functions on human skin has never been reported.

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Figure 1. 2-D PAGE images of A375 human malignant melanoma cells between control (a) and 72 h treatment (b). The images in the panels are the regions showing differentially expressed spots for protein identification. The pointed arrows indicate the identified protein spots in this study.

In this study, we used 2-D PAGE to investigate the biological effects of kojic acid on the protein expression of A375 human malignant melanoma cells through its effect on melanocytic tumorigenesis for cancer therapy and other related side effects. In addition, the bioinformatics tools were used to search the protein ontology, category classification, according to cellular component, molecular function and biological process, and prediction of protein interaction network in order to understand the cellular response and regulatory mechanism in the protein expression level of human skin cancer cells.

Materials and Methods

Test Substances and Chemicals. Human malignant melanoma cell line A375 (CRL-1619) was obtained from ATCC (Rockville, MD). Fetal bovine serum (FBS), sodium pyruvate, antibiotic-antimycotic and TRIzol reagent were purchased from Invitrogen (Carlsbad, CA). Dulbecco's Modified Eagle's Medium (DMEM), DPBS and Trypsin-EDTA were purchased from Atlanta Biologicals (Norcross, GA). RNeasy Mini Kit was purchased from Qiagen (Valencia, CA). Kojic acid and sodium bicarbonate were purchased from Sigma (St. Louis, MO). Immobiline DryStrips (IPG strips), carrier ampholytes (IPG buffers), ammonium persulfate and TEMED were purchased from GE Healthcare (Björksgatan, Uppsala, Sweden). Urea and CHAPS were purchased from J.T. Baker (Phillipsburg, NJ). Iodoacetamide and glutaraldehyde were purchased from Fluka (Industriestrasse, Buchs, Switzerland). Dithioerythritol (DTE) was purchased from AppliChem (Darmstadt, Germany). SYPRO Ruby was purchased from Molecular Probes (Eugene, OR). All primary and secondary antibodies were purchased from Abcam, Inc. (Cambridge, CA).

Cell Culture and Sample Preparation. A375 human malignant melanoma cells were used in this study. The conditions of cell culture and treatment were the same as our previous report.¹⁴ Briefly, the cells were treated with kojic acid at 8 μ g/

mL for 24, 48, and 72 h. The experiment of kojic acid treatment was done in triplicate.

Two-Dimensional Polyacrylamide Gel Electrophoresis (2-D PAGE). A375 cells were resuspended in 350 μ L of a lysis buffer containing 7 M Urea, 4% CHAPS, 2 M Thiourea, 65 mM DTE and 0.5% IPG buffer pH 3–10NL. For the first dimensional isoelectric focusing (IEF), the protein solutions (150 μ g protein) were rehydrated overnight with the commercial IPG strips (GE Healthcare, 18 cm, pH 3–10NL). Subsequently, isoelectric focusing (IEF) was carried out for 70 kVh using IPGphor IEF system (GE Healthcare) at 20 °C following the manufacturer's instruction. Prior to the second dimension, the IPG strips were equilibrated by two equilibration steps.¹⁵ The strips were loaded and run on 12.5% polyacrylamide slab gel (18 $\times$ 18 cm²) using the Protean II xi Cell system (Bio-Rad) with a programmable power control, at 45 mA/gel at 15 °C until the dye front reached the gel bottom. The separated proteins were visualized with SYPRO Ruby dye. The 2-D gel images were scanned using a Typhoon 9200 laser scanner (GE Healthcare) and exported to the image analysis software program, using PDQuest software package version 7.1.1 (Bio-Rad). Changes in fold expression of proteins were compared between control and 72 h of kojic acid treatment.

In-Gel Tryptic Digestion. The differentially expressed protein spots were excised from 2-D gels and digested with trypsin as previously reported.¹⁵ Peptides were extracted twice with 50 μ L of 50% acetonitrile (ACN)/5% trifluoroacetic acid (TFA); the extracted solutions were then combined and dried with a Speed-Vac concentrator. The peptide pellets were resuspended with 10 μ L of 0.1% TFA and purified using ZipTipC18 (Millipore, Bedford, MA). The peptides were finally eluted with 5 μ L of 75% ACN/0.1% formic acid.

Mass Spectrometric Analysis. The trypsinized samples were premixed in a ratio of 1:1 with the matrix solution (5 mg/mL α -cyano-4-hydroxycinnamic acid (CHCA) in 50% ACN, 0.1% (v/

Figure 2. 3-D views of some protein spots obtained from the experiment. These proteins were found to be correlated with p53 or involved with tumor biology. Labels: (a) control, (b) 24 h treatment, (c) 48 h treatment and (d) 72 h treatment. The protein names according to spot number are HS90B, ENPL, GRP75, VIME (nos.13, 14, 15) and 2AAA (no. 19). Intensities of the 3-D spots were analyzed by PDQuest software package version 7.1.1.

v) TFA and 2% (w/v) ammonium citrate) and spotted onto the 96-well MALDI sample stage. The samples were analyzed by the Q-TOF Ultima MALDI instrument (M@LDI; Micromass, Manchester, U.K.), as described previously.¹⁵ The MASCOT (<http://www.matrixscience.com>) search engine was used for peptide mass fingerprinting (PMF) and MS/MS ions search. Search parameters were monoisotopic mass, oxidized at methionine residues and carbamidomethylated at cysteine residues. Only one missed trypsin cleavage was allowed, and peptide mass tolerances of 50 ppm were used for PMF and MS/MS ions search. The search of identified proteins was performed using the Swiss-Prot database.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). After A375 cells treated with kojic acid at 24, 48 and 72 h were collected, total RNA was extracted by a modified method using TRIzol reagent combined with RNeasy Mini Kit, according to the manufacturer's instruction. The total RNA was quantified by the NanoDrop ND-1000 UV-vis Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). The sequences of primers used were (1) HS90B, forward 5'-GGTGGTGCTGCTGTTTAAA-3' and reverse 5'-AAGTGCAGCATTGGGTTTCCT-3'; (2) ENPL, forward 5'-ATGGAGATCCTGGAATAGGCA-3' and reverse 5'-GCTTTTCTCCAATCAAATGGC-3'; (3) GRP75, forward 5'-CGACACAGAAACCAAGATGGAA-3' and reverse 5'-AAGAGAGGATGCTGCCTGTCTAA-3'; (4) pyruvate kinase M2, forward 5'-GAAGCCT-

GTCATCTGTGCTACTCA-3' and reverse 5'-AGGATAGTCCCCTTTGGCTGTT-3'; (5) 2AAA, forward 5'-ATGCTACCCACGGTCTCTGC-3' and reverse 5'-ACAGAACAGTCAGAGCCTCCT-3'. PCR was carried out with a Px2 Thermal Cycler (Thermo Fisher Scientific, CA). The PCR condition was to heat at 95 °C for 1 min, followed by denaturation at 95 °C for 1 min, annealing at 52–55 °C for 45 s, extension at 72 °C for 1 min for 25 cycles, and finally incubation at 72 °C for 10 min. The PCR products were analyzed on 1.5% agarose gel.

Western Blotting. After SDS-PAGE analysis of control and treated samples under reducing conditions, the proteins on gel were electroblotted onto PVDF membrane using a semidry apparatus (GE Healthcare). The PVDF membrane was reacted with anti-human GRP75, 2AAA, or VIME monoclonal antibodies (Abcam; dilution 1:2000) as the primary antibody and with HRP-conjugated monoclonal anti-mouse IgG antibody (Abcam; dilution 1:2000) as the secondary antibody. β -Actin was used as control protein (dilution 1:3000). Reactive protein bands were then visualized by enhanced chemiluminescence Western blotting detection system using ECL Kit (GE Healthcare). Western blot analysis of each protein was duplicated.

Statistical Analysis. Analysis of proteins regulation changed upon kojic acid treatment was done on three independent experiments. Each set of 2-D PAGE gels was compared by image comparison software separately. The thresholds of up-

Table 1. Protein Identification of Differentially Expressed Proteins in Kojic Acid Treated A375 Cells by MALDI-TOF MS and MS/MS

spot no.	protein name	accession no.	MW (kDa) and pI		MS method	Mowse score	% coverage	no. of matched peptides	regulation (fold-change) ^a
			Apparent	Theoretical					
1	Heat shock protein HSP 90-beta (HS90B)	P08238	97.00, 4.20	83.2, 4.97	MS, MS/MS	122, 95	34, 3	17, 2	-2.50
2	Heat shock 70 kDa protein (GRP78)	P11021	91.80, 4.20	72.3, 5.07	MS, MS/MS	78, 292	28, 12	12, 5	-2.50
3	Endoplasmic precursor (Heat shock protein 90 kDa beta member 1)(ENPL)	P14625	100.00, 4.00	92.4, 4.76	MS, MS/MS	176, 166	29, 4	24, 3	-1.51
4	Heat shock cognate 71 kDa (Heat shock 70 kDa protein 8)(HSP7C)	P11142	86.64, 4.67	70.85, 5.37	MS, MS/MS	144, 112	34, 6	18, 3	-2.00
5	Stress-70 protein, mitochondrial precursor (75 kDa glucose-regulated protein) (GRP75)	P38646	89.22, 5.10	73.64, 5.87	MS, MS/MS	127, 64	29, 4	17, 2	-2.21
6	T-complex protein 1 subunit epsilon (TCP-1-epsilon)(TCPE)	P48643	76.32, 5.10	59.63, 5.45	MS	58, -	26, -	12, -	-2.20
7	Endoplasmic precursor (Heat shock protein 90 kDa beta (ENPL)	P14625	68.00, 3.95	92.41, 4.76	MS	83, -	21, -	14, -	-1.10
8	Protein disulfide-isomerase A6 precursor (PDIA6)	Q15084	63.00, 4.60	48.09, 4.95	MS, MS/MS	93, 172	30, 15	12, 4	-2.00
9	Protein disulfide-isomerase precursor (PDIA1)	P07237	71.20, 4.00	57.08, 4.76	MS, MS/MS	121, 244	38, 13	15, 4	-2.10
10	Nucleolin (NUCL)	P19338	102.00, 4.00	76.57, 4.60	MS, MS/MS	92, 40	27, 3	14, 2	-2.20
11	Vimentin (VIME)	P08670	103.00, 4.40	53.62, 5.06	MS, MS/MS	128, 143	46, 8	16, 3	-2.25
12	Vimentin (VIME)	P08670	58.50, 4.25	53.62, 5.06	MS, MS/MS	182, 123	48, 9	19, 3	-2.15
13	Vimentin (VIME)	P08670	57.00, 4.22	53.62, 5.06	MS, MS/MS	90, 103	36, 5	12, 2	-2.10
14	Vimentin (VIME)	P08670	63.00, 4.34	53.62, 5.06	MS, MS/MS	113, 43	41, 3	14, 1	-2.10
15	Vimentin (VIME)	P08670	62.50, 4.40	53.62, 5.06	MS, MS/MS	207, 137	57, 8	21, 3	-2.00
16	Vimentin (VIME)	P08670	61.30, 4.30	53.62, 5.06	MS, MS/MS	153, 99	50, 5	17, 2	-2.15
17	Gamma-enolase (EC 4.2.1.11) (2-phospho-D-glycerate hydro-lyase) (ENOG)	P09104	58.00, 4.17	47.24, 4.91	MS, MS/MS	107, 48	38, 6	14, 2	-2.00
18	Pyruvate kinase isozymes M1/M2 (KPYM)	P14618	71.16, 4.40	57.90, 7.96	MS, MS/MS	51, 53	23, 3	9, 1	+2.15
19	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform (2AAA, PP2A)	P30153	76.30, 4.25	65.18, 4.96	MS	82, -	33, -	13, -	+1.52
20	Ribonuclease inhibitor (Ribonuclease/ angiogenin inhibitor 1)(RINI)	P13489	58.50, 3.90	49.94, 4.71	MS/MS	-, 70	-, 6	-, 2	-2.18
21	Tubulin beta chain (TBB5)	P07437	65.00, 4.40	49.64, 4.78	MS, MS/MS	114, 134	33, 7	14, 2	-0.99
22	Melanoma-associated antigen 4 (MAGA-4)	P43358	55.00, 3.90	34.91, 4.68	MS/MS	-, 84	-, 5	-, 1	-2.30
23	Annexin A11 (Calycylin-associated annexin 50) (ANX11)	P50995	71.20, 6.18	54.36, 7.53	MS	68, -	28, -	11, -	-1.55
24	Eukaryotic translation initiation factor 3 subunit 2 (eIF-3 beta) (eIF3 p36) (eIF3i) (IF32)	Q13347	48.00, 5.10	36.48, 5.38	MS, MS/MS	68, 72	25, 3	9, 1	-1.00
25	60S acidic ribosomal protein P0 (L10E) (RLAO)	P05388	45.50, 5.15	34.25, 5.71	MS	98, -	41, -	11, -	-0.95
26	Inosine-5'-monophosphate dehydrogenase 2 (EC 1.1.1.205) (IMDH2)	P12268	71.16, 6.00	55.77, 6.44	MS, MS/MS	77, 75	23, 7	11, 3	-2.00
27	Aldehyde dehydrogenase 1A3 (EC 1.2.1.5) (AL1A3)	P47895	72.00, 6.25	56.07, 6.99	MS, MS/MS	81, 56	28, 2	12, 2	-1.02
28	Aldehyde dehydrogenase 1A3 (EC 1.2.1.5) (AL1A3)	P47895	72.00, 6.35	56.07, 6.99	MS, MS/MS	64, 101	21, 6	10, 3	-0.99
29	ATP synthase subunit beta, mitochondrial precursor (EC 3.6.3.14) (ATPB)	P06576	64.00, 4.50	56.52, 5.26	MS, MS/MS	124, 199	42, 16	16, 5	-1.00
30	Rab GDP dissociation inhibitor alpha (Rab GDI alpha) (GDIA)	P31150	76.32, 4.30	50.55, 5.00	MS, MS/MS	116, 55	38, 7	15, 2	-0.97

^a Regulations (fold-changes) of differentially expressed proteins are expressed at 72 h treatment of kojic acid.

regulated proteins and down-regulated proteins were 1.5- and 0.9-fold changes, respectively. Differentially expressed protein spots, which were found in all sets of experiment, in particular in comparison to 72 h treatment, were selected for protein identification.

Bioinformatics Tools for Protein Searches. For the bioinformatics tools to search protein ontology, we used the combination of databases to gain information on protein annotation (name, accession number and symbol), family and

superfamily clusters, similar protein, protein encoded gene, category classifications of cellular component, molecular function and biological process, and references. The protein search programs were used the following sequential order of databases: NCBI (<http://www.ncbi.nlm.nih.gov>), Swiss-Prot/TrEMBL (<http://www.expasy.ch/sprot>) and Proteome (<http://www.proteom.com/databases/HumanPD/reports>). In addition, the functional protein association networks or protein interactions were searched by STRING database (<http://string.embl.de/>).

Table 2. Category Classification of Differentially Expressed Proteins in Kojic Acid Treated A375 Cells and Their Interaction Partners

spot no.	protein name	cellular component	molecular function	biological process	predicted protein interaction partners
1	Heat shock protein HSP 90-beta (HS90B)	•cytoplasm •melanosome stage 1–4	•nitric-oxide synthase regulator activity •TPR domain binding	•positive regulation of nitric oxide biosynthetic process •response to unfolded protein	•TP53/p53
2	Heat shock 70 kDa protein (GRP78)	•cell surface •endoplasmic reticulum lumen •ER-Golgi intermediate compartment •membrane •nucleus •perinuclear •cytoplasm •melanosome stage 1–4	•ATP binding •calcium ion binding •caspase inhibitor activity •protein binding, bridging •unfolded protein binding	•anti-apoptosis	•ENPL •PDIA6 •GRP75 •p53
3,7	Heat shock protein 90 kDa beta member 1(ENPL)	•cytosol •microsome •endoplasmic reticulum lumen and membrane •perinuclear region of cytoplasm •cytoplasm •melanosome stage 1–4	•calcium ion binding •low-density lipoprotein •receptor binding •RNA binding •virion binding	•anti-apoptosis •protein transport •response to hypoxia •sequestering of calcium ion	•GRP75 •GRP78•PDIA4 •receptor tyrosinase-protein kinase erbB-2
4	Heat shock cognate 71 kDa (HSP7C)	•cell surface •intracellular •melanosome stage 1–4	•ATPase activity •protein binding	•protein folding, response to unfolded protein	•Bcl-2 binding •athanogene-1, –3 •HSP86 •HSP60 •p53 •DnaJ homologue subfamily A and B members •HSP90B1
5	Stress-70 protein, mitochondrial precursor (GRP75)	•cell surface •mitochondrion	•unfolded protein binding	•antiapoptosis	•T-complex protein 1 different subunits
6	T-complex protein 1 subunit epsilon (TCP-1-epsilon) (TCPE)	•cytoplasm	•protein binding	•protein folding •mammalian cell cycle progression •cell mobility •protein folding	•Beta-Actin •ERp31 •GRP78
8	Protein disulfide-isomerase A6 precursor (PDIA6)	•endoplasmic reticulum •ER-Golgi intermediate compartment •melanosome stage 1–4	•protein binding •protein disulfide isomerase activity		
9	Protein disulfide-isomerase precursor (PDIA1)	•cell surface •endoplasmic reticulum •ER-Golgi intermediate compartment •extracellular region	•procollagen-proline 4-dioxygenase activity •protein binding •protein disulfide isomerase activity	•peptidyl-proline hydroxylation to 4-hydroxy-L-proline	•ERp31 •GRP78
10	Nucleolin (NUCL)	•extracellular region •cell cortex •nucleolus	•protein C-terminus binding •RNA binding	•angiogenesis	•DNA topoisomerase •p53 •proliferation-associated protein 2G4 •RAF-1 •proteosome subunit alpha type1 •protein kinase N1 •pyruvate kinase isoenzyme M1/M2 •enzymes in glycolytic pathway
11,12,13,14,15,16	Vimentin (VIME)	•cytoplasm•intermediate filament	•protein binding •structural constituent of cytoskeleton	•cell motility	•alpha enolase •beta enolase •pyruvate dehydrogenase E1 subunits •RAF proto-oncogene serine/threonine kinase •serine/threonine protein phosphatase isoforms •protein KIAA0889
17	Gamma-enolase(ENOG)	•cytoplasm	•phosphopyruvate hydratase activity	•glycolysis	
18	Pyruvate kinase isozymes M1/M2 (KP YM)	•cytoplasm	•protein binding •pyruvate kinase activity	•glycolysis •cell proliferation	
19	Serine/ threonine-protein phosphatase 2A (2AAA, PP2A)	•cytoplasm •membrane •microtubule •cytoskeleton, •mitochondrion •nucleus •protein phosphatase type 2A complex	•antigen binding, •protein heterodimerization activity •protein phosphatase type 2A regulator activity	•ceramide metabolic process •inactivation of MAPK activity •induction of apoptosis •negative regulation of cell growth •regulation of cell adhesion •regulation of cell differentiation •regulation of progression through cell cycle •regulation of transcription •second-messenger-mediated signaling •response to organic substance	

Table 2. Continued

spot no.	protein name	cellular component	molecular function	biological process	predicted protein interaction partners
20	Ribonuclease/angiogenin inhibitor 1 (RINI)	•angiogenin-PR1 complex	•protein binding •ribonuclease inhibitor activity	•mRNA catabolic process •regulation of angiogenesis	•TNF- α •TNF-R1 •NF-kappa-B-essential modulator
21	Tubulin beta chain (TBB5)	•cytoskeleton	•MHC class I protein binding	•cell motility •natural killer cell mediated cytotoxicity	•kinesin-like protein (KIF1A) •mitogen-activated protein kinase 10 •dual specificity protein kinase TTK
22	Melanoma-associated antigen 4 (MAGA4)	•cytoplasm	•protein binding	•embryonal development •tumor transformation	•26S proteasome regulatory subunit p28 •B melanoma antigen 3 precursor
23	Annexin A11 (ANX11)	•cytoplasm •nuclear envelope •nucleoplasm	•phospholipid binding •protein binding	•tumor progression •immune response •melanoma progression •tumor suppression	•cancer/testis antigen 2 •programmed cell death protein 6 •40S ribosomal protein 24 •calcyclin •DNA-directed RNA polymerase III
24	Eukaryotic translation initiation factor 3 subunit 2 (IF32)	•eukaryotic translation initiation factor 3 complex	•protein binding •translation factor activity •nucleic acid binding	•regulation of translational initiation	•eukaryotic translation initiation factor subunits
25	60S acidic ribosomal protein P0 (RLA0)	•cytosolic large ribosomal subunit	•protein binding •RNA binding •structural constituent of ribosome	•translation	•ribosomal proteins •elongation factor 1-gamma
26	Inosine-5'-monophosphate dehydrogenase 2 (IMPDH2)	•cytoplasm	•IMP dehydrogenase activity •protein binding	•rate-limiting reaction for the de novo synthesis of guanine nucleotides •transformation and progression linked in cancer cells	•inosine5'monophosphate dehydrogenase 1 •GMP synthase •GMP reductase1 and 2 •AMP deaminase 1 and 3
27,28	Aldehyde dehydrogenase 1A3 (AL1A3)	•cytoplasm	•3-chloroallyl aldehyde activity	•lipid metabolic process •alcohol metabolic process •endothelial cell proliferation and vascular remodeling	•3-hydroxyisobutyrate dehydrogenase •acetyl-coenzyme A synthetase 2-like
29	ATP synthase subunit beta, mitochondrial precursor (ATPB)	•mitochondrial proton-transporting ATP synthase, catalytic core	•protein binding •transporter activity	•cellular energy provision and for efficient execution of apoptosis	•ATP synthase isoforms •NADH-ubiquinone oxidoreductase
30	Rab GDP dissociation inhibitor alpha (GDIA)	•cytoplasm	•GDP-dissociation inhibitor activity •protein binding	•signal transduction	•RAB4A •RAB5A •RAB2 •RAB11A •RAB9A

The combining pathway databases of BioCarta (<http://www.biocarta.com>), KEGG (<http://www.genome.ad.jp/kegg/pathways.html>) and the PubMed literature (<http://www.ncbi.nlm.nih.gov/PubMed>) were also used to search the correlated regulatory pathways of cellular response.

Results

2-D PAGE. To investigate the anticancer effect of kojic acid on protein expression pattern of A375 cells, A375 cells were treated with kojic acid for 24, 48 and 72 h, and were analyzed by 2-D PAGE. The 2-D gel images of the control without treatment and the kojic acid treated A375 cells are shown in Figure 1. Most of the proteins located in the pI ranges of 3.5–5.0 and 6.5–8.5 with the molecular weight of more than 25 kDa. With the use of PDQuest image analysis software, the numbers of protein spots detected in control and treatment groups were found to be about 540 spots with no

significant difference of the mean numbers among groups. Most of the differentially expressed protein spots showed at the high molecular weight area about 45–100 kDa, especially at acidic pH area. In order to study the late-stage of biological effect of kojic acid on protein expression profile in A375 cells, the time point at 72 h treatment was chosen to quantitate the protein expression changes between control and treatment groups, and the differentially expressed protein spots were used to further identify the proteins. There were 30 differentially expressed protein spots containing 2 up-regulated protein spots, in which the threshold of change in expression level was 1.5-fold, and 28 down-regulated protein spots, in which the threshold of change in expression level was 0.9-fold. In addition, the 3-D views of 7 interesting proteins, which have the biological functions involved with p53 tumor suppressor protein or involved in tumor biology,

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are shown in Figure 2. These proteins were then selected for validation by Western blot or RT-PCR.

Protein Identification of Differentially Expressed Proteins.

With the use of MASCOT protein identification search software for identifying both peptide mass fingerprinting (PMF) and MS/MS ion mass data, 30 differentially expressed protein spots were successfully identified. A list of identified proteins, Mowse score, apparent and theoretical MW and pI, number of PMF and MS/MS matched sequences, coverage and fold of change in expression level (up- or down-regulation) are shown in Table 1. Most of identified proteins were down-regulated in kojic acid treated A375 cells, when compared to control group. There were many isoforms of 5 heat shock proteins, 2 endoplasmic precursors, 2 protein disulfide isomerases, 6 vimentins and 2 aldehyde dehydrogenases, which possessed about 56.7% of those proteins and might be caused by post-translational modifications.

In addition, the category classification of differentially expressed proteins in kojic acid treated A375 cells, according to cellular component, molecular function and biological process, and the prediction of protein interaction partners are shown in Table 2. When considering their subcellular location, the identified proteins were found to be located at different locations in the cell component. The proteins have diverse functions, especially in protein binding, and play key roles in virtually all biological processes, including apoptosis, mammalian cell cycle progression, regulation of angiogenesis, regulation of cell proliferation, regulation of cell differentiation, and immune response. Interestingly, 11 identified proteins (36.7% of total proteins), containing heat shock proteins (HS90B, GRP75, GRP78, ENPL (x2), HSP7C), T-complex protein 1 subunit epsilon (TCPE), protein disulfide isomerases (PDIA6, PDIA1), nucleolin (NUCL) and annexin A11 (ANX11), functioned as chaperone proteins, which were found in melanosome from stage I to stage IV with different functions.

Validations by Western Blotting and RT-PCR. Among identified proteins, GRP75, VIME and serine/threonine-protein phosphatase 2A (2AAA) were validated by Western blotting, while GRP75, 2AAA, heat shock protein HS90B, endoplasmic precursor (ENPL) and pyruvate kinase isozyme M1/M2 (KPYM) were validated by RT-PCR (Figure 3). The results of protein and gene expression levels of 7 differentially expressed proteins were in agreement with the 2-D PAGE expression data. GRP75, VIME, HS90B and ENPL were down-regulated proteins in kojic acid treated A375 cells, while 2AAA and KPYM were up-regulated proteins in kojic acid treated A375 cells.

Correlation of Differentially Expressed Proteins with Tumorigenesis. Combining the protein interaction and pathway databases of STRING, BioCarta, KEGG and the PubMed literatures, we built a hypothetical model of protein interaction network in the regulation of apoptosis in tumorigenesis, in which 14 differentially expressed proteins of ANX11, ENOG, ENPL, GRP75, GRP78, HSC7C, HS90B1, KPYM, MAGA4, NUCL, PDIA6, PDI, RINI and VIME in kojic acid treated A375 cells are correlated in this network (Figure 4). This network can be divided into 4 network groups: stress related proteins, proteins involved in melanogenesis, proteins involved in regulation of apoptosis and proteins involved in energy metabolism. Most proteins were found to interact with the major regulatory proteins, such as p53 tumor suppressor protein, Bcl2 and Raf1, which play important roles in melanogenesis and regulation of apoptosis in A375 cells.

Figure 3. Validations of differentially expressed proteins by Western blot and RT-PCR analyses. For Western blotting: (a) GRP75, (b) VIME, and (c) 2AAA. For RT-PCR: (d) GRP75, (e) 2AAA, (f) HS90B (g) ENPL and (h) KPYM. Labels: c1, c2 and c3 indicate the control groups at 24, 48 and 72 h, respectively, and d1, d2 and d3 indicate the treatment groups at 24, 48 and 72 h, respectively.

Discussion

According to our previously study that investigated the toxicogenomic effects of kojic acid on gene expression profiling of A375 cells using microarray, the cell growth inhibition of A375 cells was not strongly affected by all concentrations of kojic acid and a longer treatment time at 72 h.¹⁴ There was no morphological change of A375 cells along 72 h in the presence of 8 $\mu\text{g/mL}$ kojic acid, which was referred as the mild concentration or recommended dosage of kojic acid for human skin safety. The early stage of stimulation effect of kojic acid on gene expression profiling of A375 cells also showed some tumor suppressor genes in A375 cells. Although one gene makes one protein, the modifications of proteins leading to changes in biological and physiological functions may not be caused by gene modification. In order to examine the effects of kojic acid on protein expression level in A375 cells, the same concentration of kojic acid (8 $\mu\text{g/mL}$) as in a previous study, which is under the safety recommendation in human skin care products, and various treatment times at 24, 48 and 72 h, were used in this study.

After 2-D PAGE, 30 differentially expressed proteins, showing the significant fold-change of protein expression level at 72 h treatment, were identified. We used the combined databases to propose the protein interaction network, in which 16 differentially expressed proteins are correlated to the regulation of apoptosis via the major signaling proteins, such as p53, Ras, MEK/ERK, RAF-1 and Bcl-2. Interestingly, 11 chaperone proteins were down-regulated in kojic acid treated A375 cells and were found to be correlated by their interactions to each other

Figure 4. Proposed interaction network of 15 differentially expressed proteins. The interaction network includes both direct and indirect interactions. The green spots indicated proteins that were identified in this study and the red spots indicated proteins that were not identified in this study but were found to interact with the identified proteins. Network groups: (a) stress related proteins, (b) proteins involved in melanogenesis pathway, (c) proteins involved in regulation of apoptosis, and (d) proteins involved in energy metabolism.

(Figure 4a). It is well-known that heat shock proteins are required for cell survival during stress and also have key functions in controlling cellular metabolism.^{16–18} In cancer biology, heat shock proteins were found to express at high levels in facilitate tumor cell growth and survival.¹⁹ In addition, GRP75, GRP78 and HSP90 have interaction with p53 tumor suppressor.^{20–23} HS90B has a principal role in regulation of mitogenesis and cell cycle progression and could interact with HSP70 or GRP75 to participate in the protein degradation process and protection from programmed cell death.²⁴ HSP7C interact with Bcl-2 binding athanogene-1, Bcl-2 binding athanogene-3 and other chaperone proteins, including HS90B and ENPL.²⁵ According to our results of down-regulated heat shock proteins in kojic acid treated A375 cells, the cellular stress of the malignant melanoma cells occurred under kojic acid treatment. Kojic acid could suppress the expression of heat shock proteins, which support the tumor growth, and may lead to antiapoptosis effect. On the other hand, since a primary mechanism for heat shock protein's regulation in normal cells involves the tumor repressor p53 and the related protein p63, the down-regulation of heat shock proteins may be involved in the suppression of p53 expression. It has also been reported that p53 and p63 proteins suppressed the transcription of heat shock proteins encoded genes via their binding sites for the transcription factor NF- κ B that are presented in their promoters.^{26,27} The suppression of heat shock protein expression by kojic acid may play an important role in the antitumor activity.

The 70 kDa heat shock cognate protein (HSP7C) is a member of HSP70 heat shock proteins. HSC/HSP70 function is regulated

by the chaperone Bcl-2-associated gene product-1 (BAG-1), which acts as a coupling factor between HSC/HSP70 and proteasome component.²⁸ The BAG-family proteins also contain the BAG-domain that mediates direct interaction with HSP70, ATPase domain and a number of client proteins, including the protein kinase Raf-1.^{29–31} The Raf-1 kinase is an important signaling molecule, functioning in the Ras pathway to transmit mitogenic, differentiative, and oncogenic signals to the downstream kinases MEK and ERK.³² According to BioCarta pathway of melanogenesis, Raf-1, Ras, MEK and ERK proteins are important regulatory molecules in melanocyte. Moreover, HSP70 also has important role in cotranslational maturation pathway for the human type I membrane glycoprotein tyrosinase, which interacts with calreticulin and BiP/Grp78 during its ER transit.^{32,33} Thus, the HSP7C linked to the heat shock proteins can interact with the regulatory molecules in the melanogenesis pathway, in which these proteins are upstream regulators of other melanogenic enzymes, including tyrosinase and tyrosinase related protein-1.

The protein phosphatase 2AAA (or PP2A) was found to interact with the hyperphosphorylated/desensitized Raf-1.³⁴ 2AAA is required for the recycling of Raf-1 to an activation-competent state and the melanosome aggregation.³⁵ It has interaction with the p53 tumor suppressor, Raf-1 and Bcl-2 (Figure 4b).^{36–38} 2AAA is also a major Ser/Thr phosphatase implicated in the regulation of many cellular processes, including many signal transduction pathways and cell cycle progression.^{39,40} In cancer cells, 2AAA has been proposed as a negative regulator of cellular growth and might function as a tumor suppressor.⁴¹ Confirmation by Western blot analysis

showed the up-regulated 2AAA in kojic acid treated A375 cells at 48 and 72 h. It is suggested that kojic acid can stimulate the expression of 2AAA, leading to suppression of signaling molecules in melanogenesis.

According to 2-D gel images, 6 isoforms of vimentin were down-regulated in kojic acid-treated A375 cells. It is well-known that vimentin regulates cell migration, membrane trafficking, granular secretion, protein kinase activation and regulation of stress response proteins.^{42,43} The modification of vimentin isoforms might be caused by phosphorylation, which has a central role in regulating the dynamics of vimentin assembly into polymers as well as regulating the connections between intermediated filament (IF) and IF-associated proteins.^{44,45} There are many phosphorylation processes of vimentin; for example, (i) phosphorylation by Raf-1-associated vimentin kinase resulted in the structure of vimentin filaments,⁴⁶ and (ii) phosphorylation by protein kinase C (PKC) provided many isoforms that participate in many signal transduction pathways, such as regulation of organelle movement in melanophores, tyrosine kinase, nucleotide exchange factor, serine/threonine kinase, Raf-1, PKC, cytoskeletal protein actin, proteasome component and HSC70.^{47–49} In addition to phosphorylation, the different spots of vimentin may be due to association with other molecules, such as protein kinase C, integrin or other associated proteins in transduction pathway. Change in vimentin expression level was also generated by stresses, such as heat shock and oxidative stress.^{50–52} The cleavage step of vimentin precedes the recognition of cytoskeletal filaments in apoptosis and programmed cell death mechanisms.^{53–56} Down-regulation of vimentin expression inhibits migration and invasion of colon and breast cancer cell lines.⁵⁶ According to Western blot result, the down-regulated vimentin may suppress the tumorigenesis and regulate the apoptosis of kojic acid treated A375 cells. Moreover, the presence of more than one protein bands of vimentin recognized by its monoclonal antibody may be caused by post-translational modifications.

Another protein showing tumor suppression property was pyruvate kinase isoenzyme (KPYM). It is a key sensor for energy consummation and regulation of the glycolytic pathway, in which KPYM links energy rich metabolites from the flow of glucose carbons to nucleic acid.⁵⁷ It was found to interact with gamma enolase (ENOG) (Figure 4d). In yeast cells, the over-expression of pyruvate kinases leads to an inhibition of cell proliferation by depletion of glycolytic phosphometabolites.⁵⁸ Likewise, the pyruvate kinases in eukaryotic cells are involved in fundamental processes, such as cell proliferation, tumor formation and apoptosis.^{59–61} The energy that is used by cancer cells is derived preferentially from glycolysis, in which p53 was found to modulate the balance between the utilization of respiratory and glycolytic pathway.^{62,63} According to our results of 2-D PAGE and Western blot data, the up-regulated KPYM in kojic acid treated A375 cells may play an important role in the suppression of tumor growth and metastasis.

In summary, we identified a number of differentially expressed proteins in kojic acid treated A375 cells. Most of these proteins were the key factor involved in a wide variety of cellular processes, including cell proliferation, regulation of gene expression, signaling, chromatin and cytoskeleton organization. Interestingly, our proposed interaction network revealed the correlated proteins with the regulation of apoptosis, which may lead to suppress the melanogenesis and tumorigenesis of cancer cells. Moreover, these proteins may be the useful biomarkers for further uses in diagnostic and therapeutic

applications of skin cancer. However, further functional study of these proteins may lead to better understanding of the pathogenic mechanisms and cellular response to kojic acid treatment.

Acknowledgment. This study was supported by a grant from the Chung Shan Medical University (CSMU 96-OM-B-045) and the Thematic Project in The Life Science, Academia Sinica (94C008-2).

Supporting Information Available: Table of PMF and MS/MS matched peptide sequences of identified proteins. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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PR7008737