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Molecular Profiling of A375 Human Malignant Melanoma Cells Treated with Kojic Acid and Arbutin

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1. Introduction

Malignant melanoma is a serious type of skin cancer that begins in the pigmentation system of the skin. Malignant melanomas, characterized by their high capacity for invasion and metastasis, are one of the most frequent forms of skin cancer. Primary cutaneous melanomas have been divided into four groups based on histopathology: superficial spreading melanoma, nodular melanoma, lentigo maligna melanoma and acral lentiginious melanoma[1]. Superficial spreading melanoma is the most common type of melanoma and grows outwards at first to form an irregular pattern on the skin with an uneven color. It usually starts by spreading out across the surface of the skin in what is known as the radial growth phase. Nodular melanoma occurs most often on the chest or back. It tends to grow deeper into the skin and grows quite deeply if not removed. This type of melanoma is often raised above the rest of the skin surface and feels like a bump. It may be very dark brown-black or black. Lentigo maligna melanoma is most commonly found on the face of elderly people. It grows slowly and may take several years to develop. Acral lentiginious melanoma is usually found on the palms of the hands, soles of the feet or around the toenails. Although melanoma is almost always curable in its early stage, it may be too late if the melanomas spread to other parts of the body. Early diagnosis of melanoma is therefore very important, and it is necessary to develop additional methods of melanoma therapy[2].

Malignant melanoma usually develops from the transformation and proliferation of melanocytes that normally reside in the basal cell layer of the epidermis. It also develops when the melanocytes no longer respond to normal control mechanisms of cellular growth. The melanocytes may then invade nearby structures or spread to other organs in the body (metastasis), where again they invade and compromise the function of that organ. To better understand the molecular and cellular mechanisms involved in the progression of cutaneous melanoma, human malignant melanoma cells have been widely used as a melanoma skin model for *in vitro* testing. This is because it is highly reproducible, quantifiable and easily handled in culture. Not only is it a structural cell model that closely parallels the progression of melanoma *in vivo*, it is also a cost-effective alternative to animal

and clinical testing. The antiapoptotic mechanisms regulating cell death have been implicated in conferring drug resistance in tumor cells[3]. Therefore, further knowledge on the nature of this resistance and a better understanding of the signal transduction pathways leading to tumor cell death could allow the identification of new target molecules to overcome drug resistance and improve melanoma therapy. In recent years, toxicogenomics has represented the merging of toxicology with genomics and bioinformatics. There have been significant practical challenges in pharmacology to develop both predictive and mechanism-based toxicology in an effort to identify candidate drugs and toxic agents more quickly and economically[4].

Kojic acid (5-hydroxy-2-hydroxymethyl-1,4-pyrone) is a secondary metabolic product widely used as a food additive for preventing enzymatic browning of raw crabs and shrimps and as a skin lightening or bleaching agent in cosmetic preparations[5,6]. Kojic acid is also used as an important material in antibiotic and pesticide productions and has been shown to act as a competitive and reversible inhibitor of animal and plant polyphenol oxidases, xanthine oxidase, and D- and some L-amino acid oxidases[7,8]. Acute or subchronic toxicity resulting from an oral dose of kojic acid has never been reported, but convulsions may occur if kojic acid is injected. Continuous administration of high doses of kojic acid in mice resulted in thyroid adenomas in both sexes[9]. Moreover, some reports have evaluated the tumorigenic potential of kojic acid, and the genotoxic risk for humans using kojic acid as a skin-lightening agent has also been studied[10]. However, kojic acid at high doses has certain side effects. For example, it affects thyroid function when given at a massive dose or in a long administration period by inhibiting iodine organification in the thyroid, decreasing triiodothyronine (T3) and thyroxine (T4) levels and increasing thyroid-stimulating hormone (TSH)[11]. Recently, kojic acid was found to be a tumor promoter and an enhancer of hepatocarcinogenesis in rats and in mice[12]. However, the topical use of kojic acid as a skin-lightening agent results in minimal exposure that poses a negligible or no risk of genotoxicity or toxicity to the consumer.

Arbutin, a natural compound of hydroquinone beta-D-glucopyranoside, is widely used as an ingredient in skin care products[13]. It is effective in the treatment of various cutaneous hyperpigmentations and inhibits melanogenesis in melanoma cells[14]. However, recent findings have raised serious concerns regarding both the safety and side effects of arbutin. Although the mechanisms of some inhibitory effects of arbutin on melanogenesis in melanoma cells have been elucidated, the comprehensive study of its biological effects on the regulation of malignant melanogenesis through the functional effect on carcinogenesis is not clear and rarely reported on a human genomics level. The general risk factors for melanoma include sun exposure, fair skin that burns easily, blistering sunburn, previous melanoma, previous nonmelanoma skin cancer, family history of melanoma, large numbers of moles and abnormal moles. During the progression of melanoma, different proteolytic enzyme systems, including the plasminogen-activator system and matrix metalloproteinases, play an important role in the degradation and remodeling of the extracellular matrix and basement membranes[15]. Tyrosinase is the main enzyme regulating melanogenesis, and it catalyzes three distinct reactions in the melanogenic pathway: hydroxylation of monophenol (L-tyrosine), dehydrogenation of catechol (L-DOPA) and dehydrogenation of dihydroxyindole. By contrast, catalase is the proteolytic enzyme that regulates the removal of H_2O_2, which is a potent inhibitor of tyrosinase. Peroxidase also serves to increase eumelanin polymer formation from monomers in the presence of H_2O_2 and metal ions, especially copper, which enhance the conversion of monomers to polymers[16]. Thus, changes

in enzyme levels, including modifications in protein- and gene-expression levels, influence melanogenesis in melanomas. The complex regulatory control of the biosynthesis machinery involved in melanogenesis also includes receptor-mediated pathways activated by hormones, neurotransmitters, cytokines, growth factors and eicosanoids. Additionally, receptor-independent mechanisms are activated or modified by nutrients, micromolecules, microelements, pH, cation and anion concentrations, and the oxidoreductive potential in the physicochemical milieu. Soluble factors can reach target melanocytes by circulation, by release from nerve endings or by local production to act as positive or negative regulators of melanogenesis.

Toxicogenomic applications are increasingly used to evaluate preclinical drug safety and to explain toxicity associated with compounds at the mechanistic level. Recently, a high-throughput DNA microarray technique gained an important role in genomic research by allowing the simultaneous study of the function of thousands of genes showing differential gene expression profiles. This opened the door to the discovery of biomarkers or special gene markers intended for pharmaceutical applications and disease therapy[17]. High-throughput DNA microarray technology is a powerful tool for genomic research because it allows the study of the function of thousands of genes simultaneously, reveals different gene expression profiles and elucidates the exact mechanisms and defects in genetic aberrations. Recently, microarrays have been used to analyze gene expression profiles of human melanomas. However, only a few such analyses have been reported in A375 melanoma cells. For example, the metastasis of A375 melanoma cells has been studied in nude mice by microarray analysis[18].

Proteomics is the study of the proteome, the protein complement of the genome. The terms "proteomics" and "proteome" were coined by Marc Wilkins and colleagues in the early 1990s and mirror the terms "genomics" and "genome," which describe the entire collection of genes in an organism[19]. In fact, the proteomic approach is a powerful tool for the simultaneous determination of the protein composition of complex samples. Proteomics consists of several tools, including two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) and mass spectrometry[20]. 2-D PAGE takes advantage of two unique biochemical characteristics of proteins, combining isoelectric focusing (IEF), which separates proteins according to their isoelectric point, with SDS-PAGE, which further separates them according to their molecular mass. 2-D PAGE allows simultaneous detection and quantification of up to thousands of protein spots on the same gel[21]. Mass spectrometry has been in use for many years, but it could not be applied to macromolecules such as proteins and nucleic acids. The m/z measurements are taken on molecules in the gas phase, and the heating or other treatment needed to convert a macromolecule to the gas phase usually caused its rapid decomposition. In 1988, two different techniques were developed to overcome this problem. In one of the techniques, proteins are placed in a light-absorbing matrix. With a short pulse of laser light, the proteins are ionized and then desorbed from the matrix into the vacuum system. This process, known as matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS), has been successfully used to measure the mass of a wide range of macromolecules[22]. MALDI time of flight (TOF) MS is advantageous due to the rapid and direct analysis of complex mixtures and its tolerance to buffer salts or detergents in the samples.

Although the tumorigenic potential and some genotoxic effects of kojic acid and arbutin on human skin cell lines have been widely studied, the effect of these compounds on gene and protein expression levels that may be involved in many biological functions in human skin

has never been reported. In this study, we used DNA microarrays and 2-D PAGE to investigate the biological effects of kojic acid and arbutin on gene and protein expression profiles of A375 human malignant melanoma cells and on melanocytic tumorigenesis and other related side effects of cancer therapy. These candidate genes and proteins may consequently aid in the development of early diagnostic and therapeutic applications.

2. Toxicogenomic analysis of kojic acid-treated A375 melanoma cells

2.1 Inhibitory effect of kojic acid on A375 melanoma cells

Previous examination showed that cell growth of A375 melanoma cells was directly inhibited by increasing kojic acid concentrations[18]. After treatment with kojic acid for 72 h, the highest concentration of kojic acid (1000 μg/ml) inhibited A375 melanoma cell growth less than 40%, whereas the lower concentrations of 0.32, 1.6, 8 and 40 μg/ml kojic acid inhibited A375 melanoma cell growth less than 20% (Fig. 1).

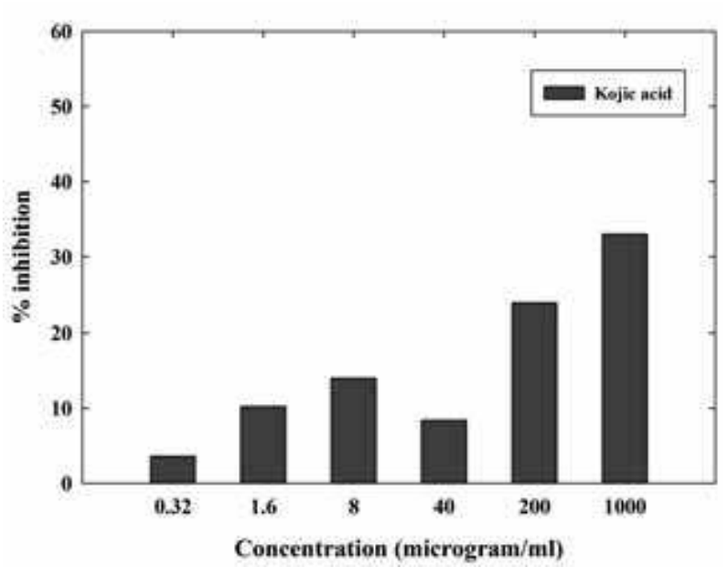

Fig. 1. Effect of different kojic acid concentrations on the growth inhibition of A375 cells at 72 h

These results indicate that the inhibition of A375 melanoma cell growth was not strongly affected by all concentrations of kojic acid. In addition, there was no morphological change of A375 melanoma cells over 24 h in the presence of 8 μg/ml kojic acid, which is a mild concentration and the recommended dosage of kojic acid for human skin safety[24]. To study the early-stage gene expression profile of A375 melanoma cells following stimulation by kojic acid, comparable to conditions used in human skin therapy, we chose the 24 h time point for the following microarray analysis.

2.2 Microarray analysis of kojic acid-treated A375 melanoma cells

Messenger RNA from control and kojic acid-treated A375 melanoma cells was labeled with Cy3- or Cy5-dCTP, respectively, using a single round of reverse transcription and hybridization with the oligonucleotide microarray chip. The microarray results showed different fluorescence intensities of Cy3 and Cy5 depending on the gene expression level[18].

Gene name	Accession No.	Ratio	Description
PSCDBP	NM_004288	15.34	Pleckstrin homology, Sec7 and coiled-coil domains
TM4SF3	NM_004616	10.99	Transmembrane 4 superfamily member 3 (TM4SF3), mRNA
KRT7	NM_005556	10.71	Keratin 7 (KRT7), mRNA
KLHL13	NM_033495	10.13	Kelch-like 13 (Drosophila) (KLHL13), mRNA
WBSCR19	NM_175064	9.58	Williams Beuren syndrome chromosome region 19 (WBSCR19)
BC031966	BC031966	8.53	cDNA clone MGC:43036 IMAGE:4839025, complete cds.
CRSP2	NM_004229	6.23	Cofactor required for Sp1 transcriptional activation, subunit 2 (CRSP2)
FGF12	NM_004113	5.95	Fibroblast growth factor 12 (FGF12), transcript variant 2, mRNA
FLJ46156	NM_198499	5.78	FLJ46156 protein (FLJ46156), mRNA
ENST00000316004	ENST00000316004	5.75	Olfactory receptor 4H12 (Fragment).
SPP1	NM_000582	5.38	Secreted phosphoprotein 1 (SPP1), mRNA
PLAGL1	NM_002656	5.14	Pleiomorphic adenoma gene-like 1 (PLAGL1)
MGC35118	NM_152453	5.07	Hypothetical protein MGC35118 (MGC35118), mRNA
TTC9	D86980	4.83	mRNA for KIAA0227 gene, partial cds.
AQP1	NM_000385	4.50	Aquaporin 1 (channel-forming integral protein, 28 kDa) (AQP1)
PHCA	NM_018367	4.47	Phytoceramidase, alkaline (PHCA), mRNA
FBXO16	NM_172366	4.21	F-box only protein 16 (FBXO16), mRNA
PARG	NM_003631	4.05	Poly (ADP-ribose) glycohydrolase (PARG), mRNA
SOAT2	NM_003578	4.02	Sterol O-acyltransferase 2 (SOAT2), mRNA
THC1991976	THC1991976	3.67	AF244540 immunodominant membrane protein precursor
PSCD3	NM_004227	3.49	Pleckstrin homology, Sec7 and coiled-coil domains 3 (PSCD3), mRNA
HIST1H2AC	NM_003512	3.34	Histone 1, H2ac (HIST1H2AC), mRNA
FLJ23018	NM_024810	3.32	Hypothetical protein FLJ23018 (FLJ23018), mRNA
PPP4R1	NM_005134	3.01	Protein phosphatase 4, regulatory subunit 1 (PPP4R1), mRNA
CPE	NM_001873	2.98	Carboxypeptidase E (CPE), mRNA
C6orf128	NM_145316	2.97	Chromosome 6 open reading frame 128 (C6orf128), mRNA
CCAR1	NM_018237	2.91	Cell division cycle and apoptosis regulator 1 (CCAR1), mRNA
FBN2	NM_001999	2.86	Fibrillin 2 (congenital contractural arachnodactyly) (FBN2), mRNA
IL22RA1	NM_021258	2.85	Interleukin 22 receptor, alpha 1 (IL22RA1), mRNA
F10	NM_000504	2.80	Coagulation factor X (F10), mRNA
GPR32	NM_001506	2.79	G protein-coupled receptor 32 (GPR32), mRNA
PDCD1LG1	NM_014143	2.65	Programmed cell death 1 ligand 1 (PDCD1LG1), mRNA
HIP1	NM_005338	2.64	Huntingtin interacting protein 1 (HIP1), mRNA
JMJD2D	NM_018039	2.61	Jumonji domain containing 2D (JMJD2D), mRNA
ARMC4	AK001238	2.57	cDNA FLJ10376 fis, clone NT2RM2001982
CAB39L	NM_030925	2.54	Calcium binding protein 39-like (CAB39L), mRNA
FLJ13089	NM_024953	2.54	Hypothetical protein FLJ13089 (FLJ13089), mRNA
OTX1	NM_014562	2.51	Orthodenticle homolog 1 (Drosophila) (OTX1), mRNA
COLQ	NM_080542	2.49	Collagen-like tail subunit of asymmetric acetylcholinesterase
PDCL	NM_005388	2.44	Phosducin-like (PDCL), mRNA
ENST00000307033	ENST00000307033	2.42	Olfactory receptor 4D5.
FLJ23311	NM_024680	2.41	FLJ23311 protein (FLJ23311), mRNA
RASSF3	AK129920	2.39	cDNA FLJ26410 fis, clone HRT09622
LOC220929	NM_182755	2.34	Hypothetical protein LOC220929 (LOC220929), mRNA
EPHA4	NM_004438	2.26	EphA4 (EPHA4), mRNA
HH114	NM_032499	2.23	Hypothetical protein HH114 (HH114), mRNA
DOCK4	AB018259	2.14	mRNA for KIAA0716 protein, partial cds.
CPNE5	NM_020939	2.11	Copine V (CPNE5), mRNA

Table 1. Up-regulated genes (48 genes, ratio > 2.0) in kojic acid-stimulated A375 cells

Analysis of gene expression changes at the RNA level using a human 1A oligonucleotide microarray complementary to 20,173 60-mer oligonucleotide probes showed a total number of 83 differentially expressed genes in kojic acid-stimulated A375 melanoma cells, including 48 up-regulated genes (Table 1, ratio > 2.0) and 35 down-regulated genes (Table 2, ratio < 0.3). We suggest that all of the differentially expressed genes may be regulated by various gene networks in the regulation of malignant tumorigenesis.

2.3 RT-qPCR validation of microarray results

Genes with a significant change in expression in the array analysis, determined by a mean degree of regulation with $p<0.01$, were chosen for RT-qPCR examination. Differentially expressed genes were grouped by biological function correlated with carcinogenesis and the significantly changed genes were selected according to the order of P values. The selected genes were validated by RT-qPCR analysis to confirm the microarray data.

RT-qPCR results showed the gene expression levels of seven expressed genes in kojic acid-stimulated A375 melanoma cells agreed with the DNA microarray expression data[18]. One gene chosen for RT-qPCR examination was the apolipoprotein B RNA editing deaminase (APOBEC1) gene[25]. The comparison of the gene expression level of sAPOBEC1 by microarray and RT-qPCR is shown in Table 3. This gene was downregulated in kojic acid-stimulated A375 melanoma cells and functions as a tumor suppressor in the regulation of carcinogenesis.

3. Toxicogenomic analysis of arbutin-treated A375 melanoma cells

3.1 Inhibitory effect of arbutin on A375 melanoma cells

A375 melanoma cell growth was directly inhibited by increasing arbutin concentrations[26]. After 72 h, the highest concentration of arbutin (1000 μg/ml) inhibited A375 cell growth up to 40%, while the lower concentrations of 0.32, 1.6, 8 and 40 μg/ml arbutin inhibited cell growth by less than 20% (Fig. 2).

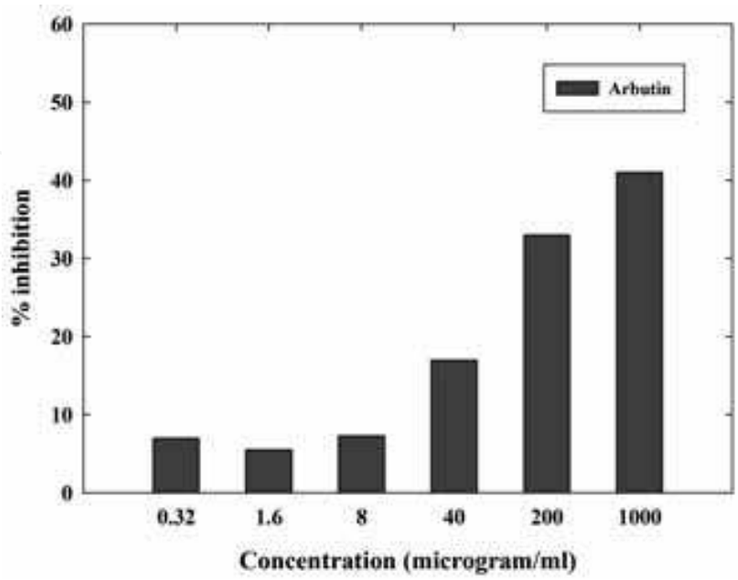

Fig. 2. Effect of arbutin concentrations on the growth inhibition of A375 cells at 72 h

Gene name	Accession	Ratio	Description
DMXL1	NM_005509	0.16	Dmx-like 1 (DMXL1), mRNA
NUDT10	NM_153183	0.16	Nudix (nucleoside diphosphate linked moiety X)-type motif 10 (NUDT10)
SAA4	NM_006512	0.17	Serum amyloid A4, constitutive (SAA4), mRNA
DSPP	NM_014208	0.17	Dentin sialophosphoprotein (DSPP), mRNA
IL6ST	NM_002184	0.18	Interleukin 6 signal transducer (gp130, oncostatin M receptor) (IL6ST)
PEX5L	NM_016559	0.18	Pex5p-related protein (PEX5R), mRNA
BC032472	BC032472	0.18	Similar to caspase 4, apoptosis-related cysteine protease
AK096677	AK096677	0.19	cDNA FLJ39358 fis, clone PEBLM2004015
FLJ14297	NM_024903	0.20	Hypothetical protein FLJ14297 (FLJ14297), mRNA
THRB	NM_000461	0.20	Thyroid hormone receptor, beta (THRB), mRNA
HLF	NM_002126	0.21	Hepatic leukemia factor (HLF), mRNA
APOBEC1	NM_001644	0.22	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1
FGF5	NM_004464	0.22	Fibroblast growth factor 5 (FGF5), transcript variant 1, mRNA
ACMSD	NM_138326	0.22	Aminocarboxymuconate semialdehyde decarboxylase (ACMSD), mRNA
SIN3B	AB014600	0.23	mRNA for KIAA0700 protein, partial cds.
KIAA0565	AB011137	0.24	mRNA for KIAA0565 protein, partial cds.
MGC34837	NM_152377	0.25	Hypothetical protein MGC34837 (MGC34837), mRNA
TAL1	NM_003189	0.25	T-cell acute lymphocytic leukemia 1 (TAL1), mRNA
SLC17A2	NM_005835	0.25	Solute carrier family 17 (sodium phosphate), member 2 (SLC17A2)
CPB1	NM_001871	0.25	Carboxypeptidase B1 (tissue) (CPB1), mRNA
USP44	NM_032147	0.26	Ubiquitin specific protease 44 (USP44), mRNA
ZNF192	NM_006298	0.26	Zinc finger protein 192 (ZNF192), mRNA
PAH	NM_000277	0.27	Phenylalanine hydroxylase (PAH), mRNA
FLJ14503	NM_152780	0.28	Hypothetical protein FLJ14503 (FLJ14503), mRNA
ZP2	NM_003460	0.29	Zona pellucida glycoprotein 2 (sperm receptor) (ZP2), mRNA
ST18	NM_014682	0.29	Suppression of tumorigenicity 18 (ST18), mRNA
PDZK10	AB002314	0.29	mRNA for KIAA0316 protein, partial cds.
TCN1	NM_001062	0.29	Transcobalamin I (vitamin B12 binding protein, R binder family) (TCN1)
ABCA13	NM_152701	0.29	ATP binding cassette gene, sub-family A (ABC1), member 13 (ABCA13)
ABCC13	NM_138726	0.29	ATP-binding cassette, sub-family C (CFTR/MRP), member 13 (ABCC13)
ADAM20	NM_003814	0.29	A disintegrin and metalloproteinase domain 20 (ADAM20), mRNA
AF090929	AF090929	0.29	Clone HQ0477 PRO0477p mRNA, complete cds.
D82326	D82326	0.29	mRNA for Na^+-independent neutral and basic amino acid transporter
LILRB5	NM_006840	0.29	Leukocyte immunoglobulin-like receptor, subfamily B, mRNA
MFAP4	NM_002404	0.29	Microfibrillar-associated protein 4 (MFAP4), mRNA

Table 2. Down-regulated genes (35 genes, ratio < 0.3) in kojic acid-stimulated A375 cells

A375 cell growth was not strongly affected by all arbutin concentrations. In addition, there were no morphological changes in cells treated with 0.32-40 μg/ml arbutin. According to the safety recommendations for a 1% prescription drug in human skin care products, the concentration of 8 μg/ml arbutin is safe and within the recommended concentration[27]. Also, there was no morphological change in the cells within 24 h in the presence of 8 μg/ml arbutin, and the inhibition of cell growth was less than 10%. However, the biological effect of arbutin on the gene expression profile in A375 melanoma cells and other genotoxic side effects have never been reported. Therefore, a purpose of this study was to investigate the genotoxic effect of arbutin on human skin and on tumorigenesis.

Gene name	P value	Microarray	RT-qPCR	(primer sequence)
APOBEC1	< 0.0001	0.22	0.12	Forward 5'-TGGATGATGTTGTACGCACTGG-3'
				Reverse 5'-TGGCGGAATCGTTTGGTAATGG-3'

Table 3. Comparison of gene expression levels of the APOBEC1 gene in kojic acid-treated A375 cells by microarray and RT-qPCR analysis

3.2 Microarray analysis of arbutin-treated A375 melanoma cells

The microarray results show different fluorescence intensities of Cy3 (control) and Cy5 (arbutin-treated A375 melanoma cells), corresponding to the expression level of thousands of genes. The differences in Cy3 and Cy5 signal intensities with a p-value of less than 0.01 (p<0.01) were considered to be significantly different[26].

Gene name	Accession	Ratio	Description
KLHL13	NM_033495	9.31	Kelch-like 13 (Drosophila) (KLHL13)
EPX	NM_000502	8.42	Eosinophil peroxidase (EPX)
TSC1	NM_000368	8.31	Tuberous sclerosis 1 (TSC1)
SCN1B	NM_001037	6.32	Sodium channel, voltage-gated, type I, beta (SCN1B)
GAGED3	NM_130777	4.54	G antigen, family D, 3 (GAGED3)
IL3RA	NM_002183	4.50	Interleukin 3 receptor, alpha (low affinity) (IL3RA)
FBXO16	NM_172366	4.22	F-box only protein 16 (FBXO16)
VPS39	NM_015289	4.10	Vacuolar protein sorting 39 (VPS39)
PARG	NM_003631	4.05	Poly (ADP-ribose) glycohydrolase (PARG)
NRP2	NM_201266	3.94	Neuropilin 2 (NRP2), transcript variant 1, mRNA
ANKRD23	NM_144994	3.65	Ankyrin repeat domain 23 (ANKRD23)
FLJ13611	NM_024941	3.61	Hypothetical protein FLJ13611 (FLJ13611)
THC1991976	THC1991976	3.55	AF244540 immunodominant membrane protein precursor
AEBP2	NM_153207	3.54	AE binding protein 2 (AEBP2)
ZBTB20	NM_015642	3.36	Zinc finger protein 288 (ZNF288)
NR2F1	NM_005654	3.27	Nuclear receptor subfamily 2, group F, member 1 (NR2F1)
KLRC4	NM_013431	3.24	Killer cell lectin-like receptor subfamily C, member 4 (KLRC4)
CAPNS2	NM_032330	3.17	Calpain small subunit 2 (CAPNS2)
APOM	NM_019101	3.12	Apolipoprotein M (APOM)
SLC23A3	NM_144712	2.65	Solute carrier family 23, member 3 (SLC23A3)
C18orf11	NM_022751	2.62	Chromosome 18 open reading frame 11 (C18orf11)
DDEF1	NM_018482	2.58	Development and differentiation enhancing factor 1 (DDEF1)
HIST1H2AC	NM_003512	2.55	Histone 1, H2ac (HIST1H2AC)
HH114	NM_032499	2.49	Hypothetical protein HH114 (HH114)
CPE	NM_001873	2.35	Carboxypeptidase E (CPE)
TARDBP	NM_007375	2.28	TAR DNA binding protein (TARDBP)
ADAM7	NM_003817	2.20	A disintegrin and metalloproteinase domain 7 (ADAM7)
KIAA1706	NM_030636	2.16	KIAA1706 protein (KIAA1706)
CCAR1	NM_018237	2.11	Cell division cycle and apoptosis regulator 1 (CCAR1)
CPNE5	NM_020939	2.10	Copine V (CPNE5)
GPR32	NM_001506	2.04	G protein-coupled receptor 32 (GPR32)
HAND1	NM_004821	2.02	Heart and neural crest derivatives expressed 1 (HAND1)

Table 4. Up-regulated genes (32 genes, ratio > 2.0) in arbutin-stimulated A375 cells

Analysis of changes in gene expression using the human oligonucleotide array revealed a total of 73 differentially expressed genes, including 32 up-regulated genes (Table 4, ratio > 2.0) and 41 down-regulated genes (Table 5, ratio < 0.3). Therefore, these genes may be useful candidate markers for early diagnostic and therapeutic applications of melanoma carcinogenesis.

Gene name	Accession	Ratio	Description
ZNF41	NM_153380	0.13	Zinc finger protein 41 (ZNF41)
F13B	NM_001994	0.16	Coagulation factor XIII, B polypeptide (F13B)
THC1910111	THC1910111	0.17	BC022679 D13Ertd275e protein
HSD3B1	NM_000862	0.17	Hydroxy-delta-5-steroid dehydrogenase, 3 beta-and steroid delta-isomerase
PF4	NM_002619	0.18	Platelet factor 4 (chemokine (C-X-C motif) ligand 4) (PF4)
IL6ST	NM_002184	0.18	Interleukin 6 signal transducer (IL6ST)
LOC162967	NM_207333	0.19	Hypothetical protein LOC162967 (LOC162967)
RFPL3	NM_006604	0.20	Ret finger protein-like 3 (RFPL3)
NTF5	NM_006179	0.20	Neurotrophin 5 (neurotrophin 4/5) (NTF5)
GPR155	NM_152529	0.20	G protein-coupled receptor 155 (GPR155)
CASP8	NM_033357	0.20	Caspase 8, apoptosis-related cysteine protease (CASP8)
C20orf17	NM_173485	0.20	Chromosome 20 open reading frame 17 (C20orf17)
AK025116	AK025116	0.20	cDNA: FLJ21463 fis, clone COL04765
MYO3B	NM_138995	0.21	Myosin IIIB (MYO3B)
ZNF167	NM_018651	0.22	Zinc finger protein 167 (ZNF167)
RFPL2	NM_006605	0.22	Ret finger protein-like 2 (RFPL2)
PDZK1	NM_002614	0.22	PDZ domain containing 1 (PDZK1)
NEGR1	NM_173808	0.22	Neuronal growth regulator 1 (NEGR1)
GPC3	NM_004484	0.22	Glypican 3 (GPC3)
DMXL1	NM_005509	0.22	Dmx-like 1 (DMXL1)
ZNF141	NM_003441	0.23	Zinc finger protein 141 (clone pHZ-44) (ZNF141)
SLC7A3	NM_032803	0.23	Solute carrier family 7, member 3 (SLC7A3)
SEMG1	NM_003007	0.23	Semenogelin I (SEMG1)
L17325	L17325	0.23	Pre-T/NK cell associated protein (1D12A2)
FMNL3	NM_175736	0.23	Formin-like 3 (FMNL3)
CPB1	NM_001871	0.23	Carboxypeptidase B1 (tissue) (CPB1)
ADAMTS9	NM_020249	0.23	A disintegrin-like and metalloprotease with thrombospondin type 1 motif
FGF5	NM_004464	0.24	Fibroblast growth factor 5 (FGF5)
C14orf148	NM_138791	0.24	Hypothetical protein FLJ32809 (LOC122945)
BMPR1B	NM_001203	0.24	Bone morphogenetic protein receptor, type IB (BMPR1B)
ABCA10	NM_080282	0.24	ATP-binding cassette, sub-family A (ABC1), member 10 (ABCA10)
PKHD1	NM_138694	0.25	Polycystic kidney and hepatic disease 1 (PKHD1)
NM_152768	NM_152768	0.25	Hypothetical protein FLJ25378 (FLJ25378)
ABCC13	NM_138726	0.25	ATP-binding cassette, sub-family C (CFTR/MRP), member 13 (ABCC13)
SLC15A3	NM_016582	0.27	Solute carrier family 15, member 3 (SLC15A3)
LGALS14	NM_020129	0.27	Placental protein 13-like protein (PPL13)
C6orf97	NM_025059	0.27	Chromosome 6 open reading frame 97 (C6orf97)
AX721299	AX721299	0.27	Sequence 259 from Patent WO0220754
C15orf26	NM_173528	0.28	Hypothetical protein FLJ38615 (FLJ38615)
ADAM20	NM_003814	0.29	A disintegrin and metalloproteinase domain 20 (ADAM20)
ACMSD	NM_138326	0.29	Aminocarboxymuconate semialdehyde decarboxylase (ACMSD)

Table 5. Down-regulated genes (41 genes, ratio < 0.3) in arbutin-stimulated A375 cells

4. Proteomic analysis of kojic acid-treated A375 melanoma cells

4.1 2-D PAGE of A375 melanoma cells following kojic acid treatment

To investigate the anticancer effect of kojic acid on the protein expression pattern of A375 melanoma cells, cells were treated with kojic acid for 24, 48 and 72 h and analyzed by 2-D PAGE[28]. The 2-D gel images of the untreated control and kojic acid-treated A375 melanoma cells are shown in Fig. 3.

Most of the proteins have p*I* ranges of 3.5-5.0 or 6.5-8.5 with molecular weights of more than 25 kDa. Using PDQuest image analysis software, approximately 540 spots were detected in control and treatment groups with no significant difference of the mean numbers among groups. Most of the differentially expressed protein spots have a high molecular weight of 45-100 kDa, especially at acidic pH.

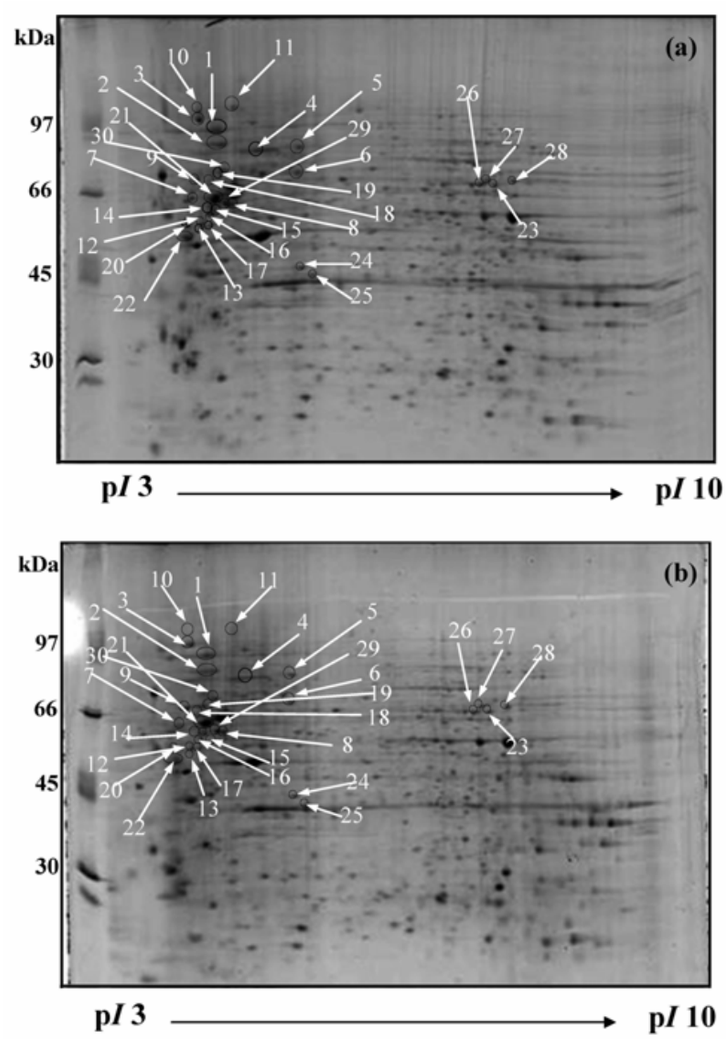

Fig. 3. 2-D PAGE images of A375 human malignant melanoma cells after control (a) and 72 h treatment (b) with arbutin. The images in the panels are the regions showing differentially expressed spots for protein identification. The pointed arrows indicate the protein spots identified in this study

To study the biological effect of kojic acid on late-stage protein expression in A375 melanoma cells, the time point of 72 h was chosen to quantify protein expression changes between the control and treatment groups, and the differentially expressed protein spots were used to further identify the proteins. There were 30 differentially expressed protein spots, including 2 up-regulated protein spots, for which the threshold of change in expression level was 1.5-fold, and 28 down-regulated protein spots, for which the threshold of change in expression level was 0.9-fold. Seven interesting proteins that have biological functions associated with the p53 tumor suppressor protein or are involved in tumor biology were then selected for validation by Western blot or RT-PCR.

4.2 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[28]. A list of identified proteins, the apparent and theoretical MW, p*I* and fold change in expression level (up- or down-regulation) are shown in Table 6. Most of identified proteins were downregulated in kojic acid-treated A375 melanoma cells when compared to the control group. There were multiple isoforms of 5 heat shock proteins, 2 endoplasmin precursors, 2 protein disulfide isomerases, 6 vimentins and 2 aldehyde dehydrogenases, which comprised about 56.7% of the identified proteins, which might be due to post-translational modifications. In addition, the prediction of protein interaction partners is shown in Table 6. The identified proteins were found at different locations within the cell. 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 the immune response. Interestingly, 11 identified proteins (36.7% of total proteins), including 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), are chaperone proteins, which were found in melanosomes from stage I to stage IV with different functions.

4.3 Validation by Western blotting and RT-PCR

Of the identified proteins, GRP75, heat shock protein HS90B, ENPL and pyruvate kinase isozyme M1/M2 (KPYM) were validated by RT-PCR, while GRP75, VIME and serine/threonine-protein phosphatase 2A (2AAA) were validated by Western blotting (Fig. 4).

Protein and gene expression levels of 7 differentially expressed proteins were in agreement with the 2-D PAGE expression data[28]. The proteins GRP75, VIME, HS90B and ENPL were downregulated in kojic acid-treated A375 melanoma cells, while the proteins 2AAA and KPYM were upregulated.

5. Proteomic analysis of arbutin-treated A375 melanoma cells

5.1 2-D PAGE of A375 melanoma cells following arbutin treatment

The 2-D PAGE images of A375 melanoma cells without treatment (control) and with arbutin treatment at 24, 48 and 72 h are shown in Fig. 5.

Sopt No.	Protein Name	Accession no.	Mr (Exp)	p*I* (Exp)	Mr (Cal)	p*I* (Cal)	Fold-change	Predicted protein interaction partners
1	Heat shock protein HSP 90-beta	P08238	97.00	4.20	83.2	4.97	-2.50	•TP53/p53
2	Heat shock 70 kDa protein	P11021	91.80	4.20	72.3	5.07	-2.50	•ENPL •PDIA6 •GRP75 •p53
3	Endoplasmin precursor (ENPL)	P14625	100.00	4.00	92.4	4.76	-1.51	•GRP75 •GRP78 •PDIA4 receptor •Tyrosinase-protein kinase erbB-2
4	Heat shock cognate 71 kDa (HSP7C)	P11142	86.64	4.67	70.85	5.37	-2.00	•Bcl-2 binding athanogene-1, -3 •HSP86 •HSP60
5	Stress-70 protein, mitochondrial precursor (GRP75)	P38646	89.22	5.10	73.64	5.87	-2.21	•p53 •DnaJ homologue subfamily A and B members •HSP90B1
6	T-complex protein 1 subunit epsilon (TCPE)	P48643	76.32	5.10	59.63	5.45	-2.20	•T-complex protein 1 different subunits
7	Endoplasmin precursor (ENPL)	P14625	68.00	3.95	92.41	4.76	-1.10	•GRP75 •GRP78 •PDIA4 receptor •Tyrosinase-protein kinase erbB-2
8	Protein disulfide-isomerase A6 precursor (PDIA6)	Q15084	63.00	4.60	48.09	4.95	-2.00	•Beta-Actin •ERp31 • GRP78
9	Protein disulfide-isomerase precursor (PDIA1)	P07237	71.20	4.00	57.08	4.76	-2.10	•ERp31 •GRP78
10	Nucleolin (NUCL)	P19338	102.00	4.00	76.57	4.60	-2.20	•DNA topoisomerase •p53 •Proliferation-associated protein 2G4
11	Vimentin (VIME)	P08670	103.00	4.40	53.62	5.06	-2.25	•RAF-1 •Proteosome subunit alpha type1 •Protein kinase N1
12	Vimentin (VIME)	P08670	58.50	4.25	53.62	5.06	-2.15	•RAF-1 •Proteosome subunit alpha type1 •Protein kinase N1
13	Vimentin (VIME)	P08670	57.00	4.22	53.62	5.06	-2.10	•RAF-1 •Proteosome subunit alpha type1 •Protein kinase N1
14	Vimentin (VIME)	P08670	63.00	4.34	53.62	5.06	-2.10	•RAF-1 •Proteosome subunit alpha type1 •Protein kinase N1
15	Vimentin (VIME)	P08670	62.50	4.40	53.62	5.06	-2.00	•RAF-1 •Proteosome subunit alpha type1 •protein kinase N1
16	Vimentin (VIME)	P08670	61.30	4.30	53.62	5.06	-2.15	•RAF-1 •Proteosome subunit alpha type1 •Protein kinase N1
17	Gamma-enolase (EC 4.2.1. 11)	P09104	58.00	4.17	47.24	4.91	-2.00	•Pyruvate kinase isoenzyme M1/M2 •Enzymes in glycolytic pathway
18	Pyruvate kinase isozymes M1/M2 (KPYM)	P14618	71.16	4.40	57.90	7.96	+2.15	•Alpha enolase •Beta enolase •Pyruvate dehydrogenase E1 subunits
19	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform (2AAA, PP2A)	P30153	76.30	4.25	65.18	4.96	+1.52	•RAF proto-oncogene serine/threonine kinase •Serine/threonine protein phosphatase isoforms •Protein KIAA0889
20	Ribonuclease inhibitor (RINI)	P13489	58.50	3.90	49.94	4.71	-2.18	•TNF-α •TNF-R1 •NF-kappa-B-essential modulator
21	Tubulin beta chain (TBB5)	P07437	65.00	4.40	49.64	4.78	-0.99	•Kinesin-like protein (KIF1A) •Mitogen-activated protein kinase 10 •Dual specificity protein kinase TTK
22	Melanoma-associated antigen 4 (MAGA-4)	P43358	55.00	3.90	34.91	4.68	-2.30	•26S proteosome regulatory subunit p28 •B melanoma antigen 3 precursor •Cancer/testis antigen 2
23	Annexin A11 (Calcyclin-associated annexin 50) (ANX11)	P50995	71.20	6.18	54.36	7.53	-1.55	•Programmed cell death protein 6 •40S ribosomal protein 24 •Calcyclin •DNA-directed RNA polymerase III
24	Eukaryotic translation initiation factor 3 subunit 2	Q13347	48.00	5.10	36.48	5.38	-1.00	•Eukaryotic translation initiation factor subunits
25	60S acidic ribosomal protein P0 (RLAO)	P05388	45.50	5.15	34.25	5.71	-0.95	•Ribosomal proteins •Elongation factor 1-gamma
26	Inosine-5'monophosphate dehydrogenase 2 (EC 1.1.1.205) (IMDH2)	P12268	71.16	6.00	55.77	6.44	-2.00	•Inosine-5'monophosphate dehydrogenase 1 •GMP synthase •GMP reductase 1 and 2 •AMP deaminase 1 and 3
27	Aldehyde dehydrogenase 1A3 (EC 1.2.1.5) (AL1A3)	P47895	72.00	6.25	56.07	6.99	-1.02	•3-Hydroxyisobutyrate dehydrogenase •Acetyl-coenzyme A synthetase 2-like
28	Aldehyde dehydrogenase 1A3 (EC 1.2.1.5) (AL1A3)	P47895	72.00	6.35	56.07	6.99	-0.99	•3-Hydroxyisobutyrate dehydrogenase •Acetyl-coenzyme A synthetase 2-like
29	ATP synthase subunit beta, mitochondrial precursor (EC 3.6.3.14)	P06576	64.00	4.50	56.52	5.26	-1.00	•ATP synthase isoforms •NADH-ubiquinone oxidoreductase
30	Rab GDP dissociation inhibitor alpha (Rab GDI alpha)	P31150	76.32	4.30	50.55	5.00	-0.97	•RAB4A •RAB5A •RAB2 •RAB11A •RAB9A

Table 6. Protein identification of differentially expressed proteins in kojic acid-treated A375 cells and the prediction of protein interaction partners

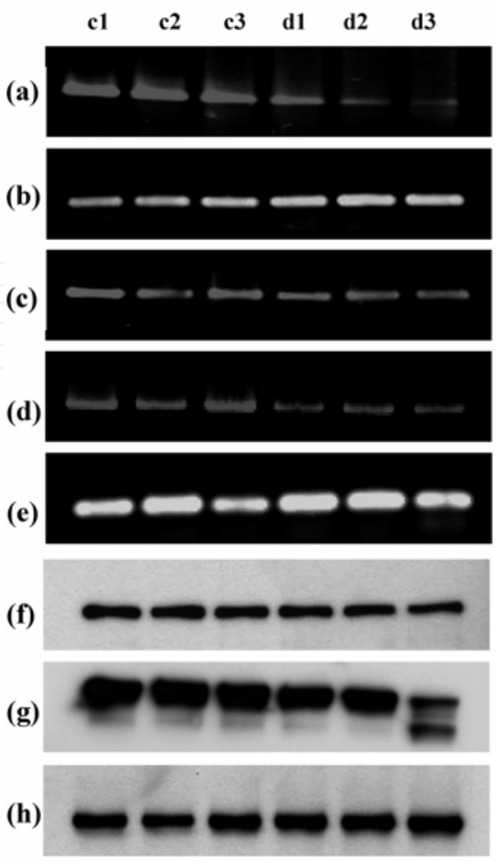

Fig. 4. Validation of differentially expressed proteins by Western blot and RT-PCR analyses. RT-PCR of (a) GRP75, (b) 2AAA, (c) HS90B (d) ENPL and (e) KPYM. Western blot of (f) GRP75, (g) VIME, and (h) 2AAA. c1, c2 and c3 indicate the control groups at 24, 48 and 72 h, respectively. d1, d2 and d3 indicate the treatment groups at 24, 48 and 72 h, respectively

The molecular weights of the proteins were distributed within the range of 25 to 97 kDa, and the differentially expressed protein spots had isoelectric points distributed between acidic and basic pH[29]. Using PDQuest image analysis software, approximately 540 spots were found in the control and treatment groups with no significant difference between the groups. There were 40 differentially expressed protein spots, including 10 up-regulated protein spots with a threshold of change in expression level of 1.0-fold, and 40 down-regulated protein spots with a threshold of change in expression level of 0.9-fold.

5.2 Identification of differentially expressed proteins

Using the MASCOT protein identification search software for identifying both PMF and MS/MS ion mass data, 26 differentially expressed proteins were successfully identified[29]. Among these proteins, there were five up-regulated and 15 down-regulated proteins (including their isoforms). Most of the identified proteins were downregulated in arbutin-treated A375 melanoma cells, and 13 isoforms of six identical proteins were observed. There were two vimentins, two heterogeneous nuclear ribonucleoproteins A2/B1, two heterogeneous nuclear ribonucleoproteins A1, two peroxiredoxin-1 proteins, three glyceraldehyde-3-phosphate dehydrogenases and two alpha-enolases, accounting for about

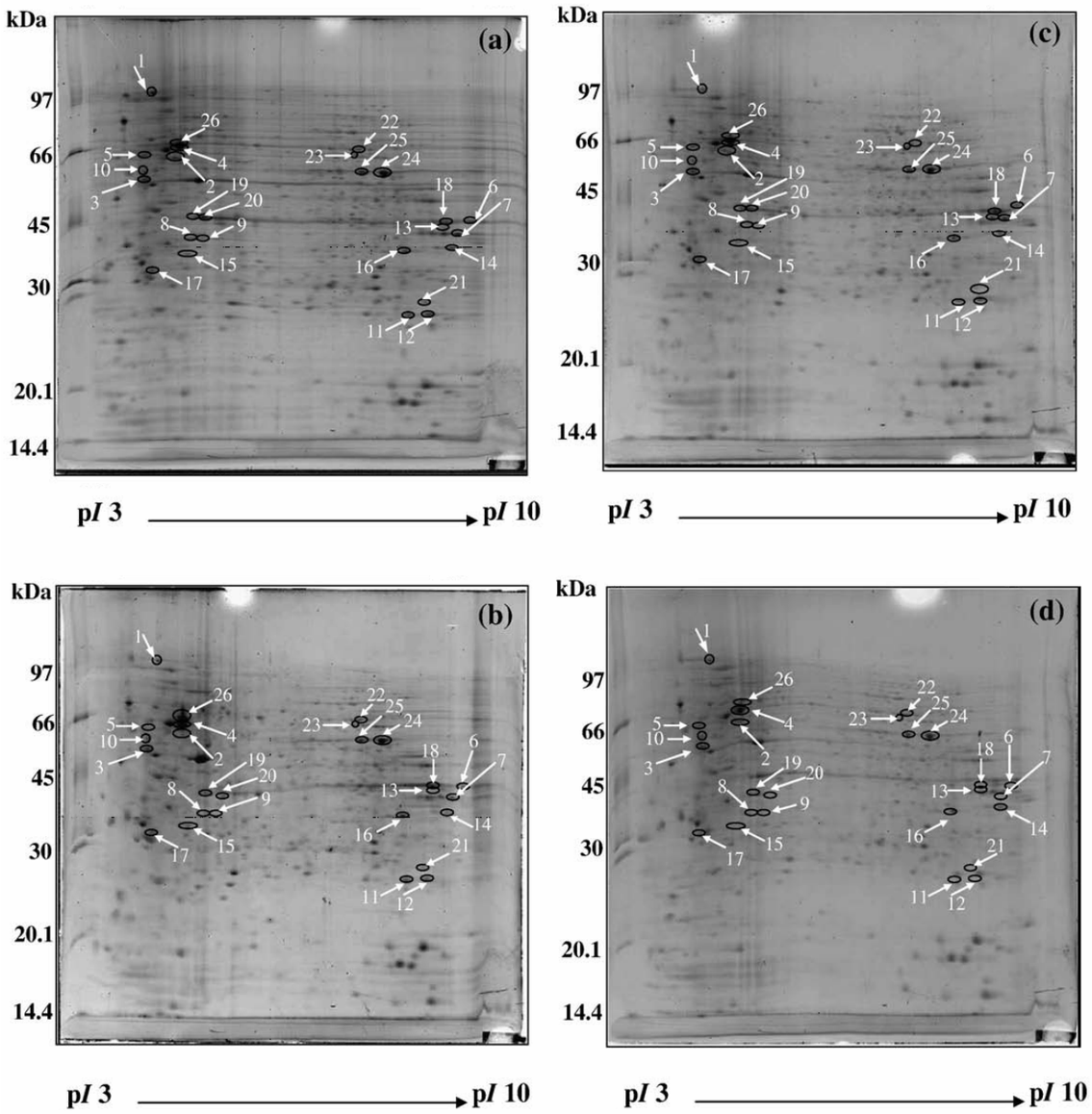

Fig. 5. 2-D PAGE images from A375 human malignant melanoma cells under the following conditions: (a) control, (b) 24-h arbutin treatment, (c) 48-h arbutin treatment and (d) 72-h arbutin treatment. The samples were separated on a homogeneous 12.5% SDS-PAGE gel using a pH 3–10 NL IPG strip. The pointed arrows indicate the protein spots identified in this study

50% of all identified proteins possibly due to post-translational modifications. The protein interaction partners are also predicted. The identified proteins are distributed to many locations within the cell, have diverse functions and play important roles in many biological processes, especially in cancer biology, such as apoptosis, angiogenesis, cell proliferation, cell differentiation and the immune response. Interestingly, five identified proteins, heat shock protein 90 kDa beta member 1 (ENPL), voltage-dependent anion-selective channel protein 1 (VDAC-1), chloride intracellular channel protein 1 (CLIC1), guanine nucleotide-

binding protein subunit beta (GBLP) and 14-3-3 protein gamma (14-3-3G) (19% of total proteins), were found to play important roles in apoptosis and signal transduction. Additionally, six identified proteins (nine spots including their isoforms, 34.6% of total proteins), nuclear ribonucleoprotein H (HNRH1), ribonucleoproteins A2/B1 (ROA2), ribonucleoprotein A1 (ROA1), ribonuclease inhibitor (RINI), 14-3-3G and alpha-enolase (ENOA), function in nucleic acid processing or transcriptional regulation. Moreover, four identified proteins (seven spots including their isoforms, 27% of total proteins), malate dehydrogenase (MDHM), glyceraldehyde-3-phosphate dehydrogenase (G3P), glucose-6-phosphate 1-dehydrogenase (G6PD) and ENOA are involved in carbohydrate metabolism pathways and have additional functions in the regulation of cell growth and maintenance of cellular functions.

5.3 Validation by Western blot or RT-PCR

Of the identified proteins, vimentin (VIME), 14-3-3G, peroxiredoxin-1 (PRDX1), ENPL, inosine-5′-monophosphate dehydrogenase 2 (IMDH2), ENOA, VDAC-1 and p53 were validated by RT-PCR (Fig. 6). The protein and gene expression levels of eight differentially expressed proteins were in agreement with the 2-D PAGE expression data[29]. 14-3-3G, VDAC-1 and p53 were upregulated in the arbutin-treated A375 melanoma cells, whereas VIME, PRDX1, ENPL, IMDH2 and ENOA were downregulated in the arbutin-treated A375 melanoma cells.

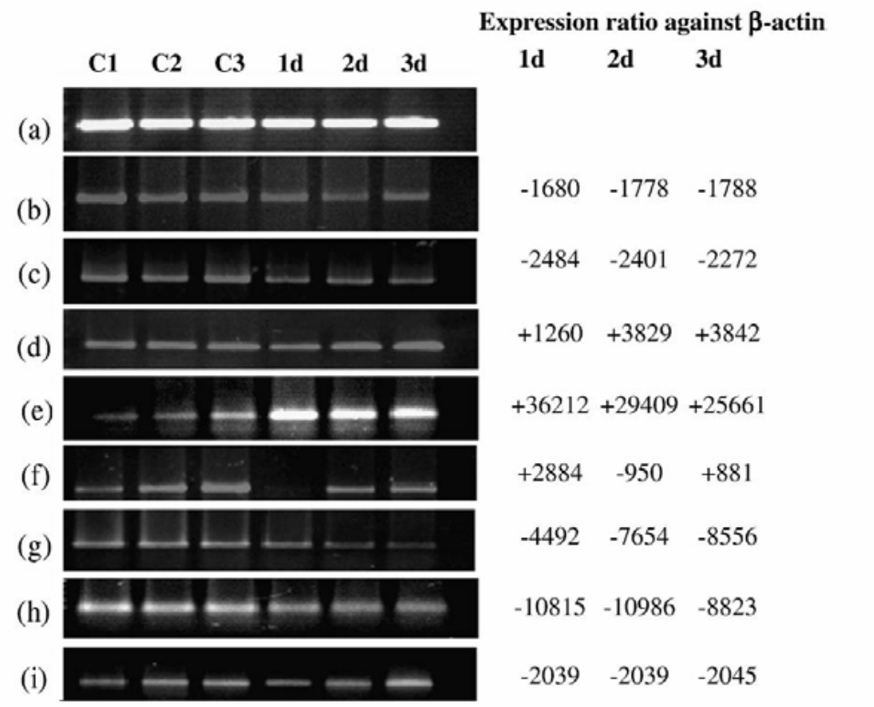

Fig. 6. Validation of differentially expressed proteins by RT-PCR analyses. (a) β-actin, (b) VIME, (c) ENPL, (d) 14-3-3G, (e) VDAC-1, (f) ENOA, (g) PROX1, (h) IMDH2 and (i) p53. C1, C2 and C3 indicate the control group at 24, 48 and 72 h, respectively. 1d, 2d and 3d indicate the treatment groups at 24, 48 and 72 h, respectively

6. Discussion

Although some of the biological applications and potential effects of kojic acid have been partially elucidated[30], the effects of kojic acid on a human genomics level, including gene regulatory mechanisms, are not clear and has rarely been reported. In this study, we used A375 human skin malignant melanoma cells to examine the genotoxicity of kojic acid in carcinoma therapy, not including its skin-whitening effect. To examine the genotoxicity effect of kojic acid on the gene expression profile of A375 melanoma cells, microarray technology providing a high-throughput method for easily screening the number of differentially expressed genes was used. It has been used for analysis of gene expression in dermatological studies, including studies of human melanomas[31]. However, the study of gene expression in kojic acid-stimulated A375 melanoma cells by DNA microarray has never been reported. It is challenging to use the high-throughput technology of DNA microarray for studying the effect of kojic acid on the large numbers of differentially expressed genes in A375 melanoma cells and its anti-tumorigenic function to regulate the malignancy of melanoma. We used the human 1A oligonucleotide microarray to analyze gene expression of human skin A375 melanoma cells for the following reasons: (i) the human 1A oligonucleotide microarray is comprised of 20,173 oligonucleotide probes that span conserved exons across transcripts of 18,716 human genes, is compatible with human skin A375 melanoma cells and could screen large numbers of differentially expressed human genes at a single time. (ii) The human 1A oligonucleotide microarray uses a convenient two-color labeling procedure that reduces experimental variability by allowing biological samples to be directly compared with each other on the same microarray after undergoing the same hybridization incubation. This microarray platform allowed the analysis of kojic acid-responding genes in A375 melanoma cells that may be used as gene markers of malignant melanoma for diagnostic and therapeutic applications in human skin. We examined the effect of various concentrations (0.32, 1.6, 8, 40, 200 and 1000 μg/ml) of kojic acid on growth inhibition of A375 melanoma cells. Not all kojic acid concentrations strongly inhibited the cell growth of A375 melanoma cells, even at the longer incubation time of 72 h. When using microarray technology to study the overall effects of kojic acid on gene expression of A375 melanoma cells, we used 8 μg/ml (0.8%) kojic acid because this is the mildest concentration that could inhibit A375 melanoma cells less than 20% while avoiding potential differences in gene expression data resulting from the cell death response due to cytotoxicity from higher concentrations of kojic acid. The safe level of kojic acid in human skin care products for general use is up to 1% without a prescription[24]. Therefore, 8 μg/ml kojic acid (0.8%) is safe for human skin and can be used to study the gene expression profile of A375 melanoma cells and identify the regulatory functions of these genes in carcinogenesis therapy. After microarray data were collected, we used bioinformatic tools to identify the differentially expressed genes and searched Gene Ontology for useful information. The information obtained can be used to further study regulatory mechanisms and gene markers for applications in early cancer diagnosis and therapy. For a number of differentially expressed genes, we selected significant genes involved in carcinogenesis and compared microarray analysis data with RT-qPCR data to validate changes in gene expression. RT-qPCR offered confirmatory quantitative results under stringent conditions. We show the quantification of kojic acid-responding genes from RT-qPCR is in agreement with the DNA microarray expression data. This indicates that the validation of genes in the robust biomarkers lists was done using precise data. These validated genes may become

useful biomarkers to understand the biochemistry of a drug/small molecule response in future clinical studies. Interestingly, we have found seven significant kojic acid-responsive genes downregulated in kojic acid-stimulated A375 melanoma cells. Most of the genes are related to the regulation of carcinogenesis. Apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1 (APOBEC1), is the central component of an RNA editosome, whose physiological role is the deamination of apoB mRNA cytidine-6666 to uracil in gastrointestinal tissues, thereby creating a premature stop codon[32]. Uncontrolled cytidine deamination could generate inappropriately folded polypeptides, dominant-negative proteins, or mutations in tumor suppressor genes and thus contribute to tumor formation[33] . Overexpression of APOBEC1 has led to the development of a number of different cancers, such as gastrointestinal cancer, colon cancer and heptocellular cancer, in a variety of tissues. This implies that inappropriate expression of a member of the DNA deaminase family could have potential oncogenic activity[34]. Furthermore, most human carcinomas are caused by APOBEC1-mediated mRNA editing[35]. Thus, down-regulated APOBEC1 may potentially deactivate the tumor progression of A375 melanoma cells.

In conclusion, we used high-throughput DNA microarray and RT-qPCR techniques for the global analysis of differentially expressed genes responding to kojic acid in A375 human melanoma cells. The differentially expressed genes were classified depending on cellular localization, molecular function and biological process, and they led to an exploration of the more valuable data on the regulation of melanoma carcinogenesis against kojic acid. In addition, seven of the down-regulated genes that showed significantly different expression in A375 melanoma cells after kojic acid treatment function as tumor suppressors and may disrupt the regulation of melanoma tumorigenesis in human malignant melanoma cells. These genes may be useful markers in further diagnostic and therapeutic applications. Moreover, our findings show the probable side effects and disadvantages of kojic acid due to its effect on the immune system, bone development and maintenance. However, we will further study the effects of kojic acid on biological and molecular mechanisms in human melanoma skin cells, including in other parts of the body, and also examine other biological functions of kojic acid in its cosmetic and/or therapeutic applications. We also examined the effect of different arbutin concentrations on the growth inhibition of A375 melanoma cells and found that the concentrations used did not strongly inhibit cell growth, even though the incubation time was up to 72 h. We used 8 μg/ml (0.8% w/v) arbutin because (i) this was the same concentration of kojic acid used[18], because this concentration was lower than the safety recommendation of 1% in prescription human skin care products[36]; (ii) this concentration inhibited cell growth less than 10% with no morphological change of cells; and (iii) it avoids changes in gene expression data resulting from the cell death response due to cytotoxicity from a high concentration of arbutin. Therefore, 8 μg/ml arbutin, which is safe for use on human skin, was used to study the genotoxic effect on the gene expression profile of A375 melanoma cells and for examining the differential gene expression and other side effects of altered signaling pathways for cancer therapy. To study the effect of arbutin on the gene expression level of A375 melanoma cells, RNA was isolated from cells 24 h after arbutin treatment to study gene expression levels with microarray analysis.

We also used high-throughput DNA microarray and bioinformatic tools in a global analysis of differentially expressed genes in arbutin-treated A375 malignant melanoma cells. These genes were classified by Gene Ontology and led to an exploration of more valuable data on the regulation of melanoma carcinogenesis. In addition, four down-regulated genes are candidate tumor suppressor genes in A375 melanoma cells following arbutin treatment.

They may disrupt the regulation of malignant tumorigenesis in human malignant melanoma cells and may be useful markers for further diagnostic and therapeutic applications. Moreover, the genotoxic effect of arbutin on the gene expression profile of A375 melanoma cells was similar to kojic acid, which indicates a similar regulation of malignant tumorigenesis by these drugs. However, we will conduct further studies on the effects of arbutin on biological and molecular mechanisms in human skin cells. We will also examine other biological characteristics of arbutin for therapeutic applications. According to our study investigating the toxicogenomic effects of kojic acid on the gene expression profile of A375 melanoma cells using microarray analysis, the cell growth inhibition was not strongly affected by all concentrations of kojic acid at a longer treatment time of 72 h[18]. There was no morphological change of A375 melanoma cells over the 72 h exposure to 8 μg/ml kojic acid, which is a mild concentration and the recommended safe dose of kojic acid for human skin. The early stage of the kojic acid stimulatory effect on the gene expression profile of A375 melanoma cells also uncovered some tumor suppressor genes. Although one gene makes one protein, protein modifications leading to changes in biological and physiological functions may not be caused by gene modifications. To examine the effects of kojic acid on protein expression levels in A375 melanoma cells, the same concentration of kojic acid (8 μg/ml) as in the microarray study, which is compliant with the safety recommendation in human skin care products, and various treatment times (24, 48 and 72 h) were used in this study. 2-D PAGE identified 30 differentially expressed proteins that showed a significant change in expression levels following 72 h of drug treatment. We used combined databases to propose a protein interaction network in which 16 differentially expressed proteins are involved in the regulation of apoptosis via major signaling proteins, such as p53, Ras, MEK/ERK, RAF-1 and Bcl-2. Interestingly, 11 chaperone proteins were downregulated in kojic acid-treated A375 melanoma cells and were found to interact with each other. It is well known that heat shock proteins are required for cell survival during stress and also have key functions in controlling cellular metabolism[37]. In cancer biology, heat shock proteins are expressed at high levels during facilitated tumor cell growth and survival[38]. In addition, GRP75, GRP78 and HSP90 interact with the p53 tumor suppressor[39]. HSP90B has a principal role in the 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. HSP7C interacts with the Bcl-2 binding proteins athanogene-1 and athanogene-3 and other chaperone proteins, including HS90B and ENPL.

According to our results of down-regulated heat shock proteins in kojic acid-treated A375 melanoma cells, cellular stress in malignant melanoma cells was observed following kojic acid treatment. Kojic acid could suppress the expression of heat shock proteins, which support tumor growth and may lead to an antiapoptosis effect. Alternatively, because a primary mechanism for the regulation of heat shock proteins in normal cells involves the tumor repressor p53 and the related protein p63, the downregulation of heat shock proteins may be involved in the suppression of p53 expression. It has also been reported that p53 and p63 proteins suppress the transcription of heat shock protein-encoding genes via their promoter binding sites for the transcription factor NF-Y[40]. The suppression of heat shock protein expression by kojic acid may play an important role in its antitumor activity. The 70-kDa heat shock cognate protein (HSP7C) is a member of the HSP70 heat shock protein family. 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 a proteasome component[41]. BAG-family proteins also contain the BAG-domain, which mediates a direct

interaction with HSP70, the ATPase domain and a number of client proteins, including the protein kinase Raf-1[42]. Raf-1 is an important signaling molecule that functions in the Ras pathway to transmit mitogenic, differentiative and oncogenic signals to the downstream kinases MEK and ERK[43]. According to the BioCarta pathway of melanogenesis, Raf-1, Ras, MEK and ERK are important regulatory proteins in melanocytes. HSP70 also has an important role in the cotranslational maturation pathway of the human type I membrane glycoprotein tyrosinase, which interacts with calreticulin and BiP/Grp78 during its ER transit[44]. Thus, the HSP7C associated with heat shock proteins can interact with 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 hyperphosphorylated/desensitized Raf-1[45]. 2AAA is required for recycling of Raf-1 to an activation-competent state and for melanosome aggregation, and it interacts with p53, Raf-1 and Bcl-2. 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[46]. In cancer cells, 2AAA has been proposed to negatively regulate cellular growth and might function as a tumor suppressor[47]. Confirmation by Western blot analysis showed 2AAA is upregulated in kojic acid-treated A375 melanoma cells at 48 and 72 h. It has been suggested that kojic acid may stimulate the expression of 2AAA, leading to suppression of signaling molecules in melanogenesis. According to 2-D gel analysis, 6 isoforms of vimentin were downregulated in kojic acid-treated A375 melanoma cells. It is well known that vimentin regulates cell migration, membrane trafficking, granular secretion, protein kinase activation and regulation of stress response proteins[48]. 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 in regulating the connections between intermediate filament (IF) and IF-associated proteins[49]. There are many phosphorylation events affecting vimentin. For example, phosphorylation by Raf-1-associated vimentin kinase results in the proper structure of vimentin filaments[50] and phosphorylation by protein kinase C (PKC) creates isoforms that participate in the regulation of organelle movement in melanophores and many signal transduction pathways involving tyrosine kinase, nucleotide exchange factor, serine/threonine kinase, Raf-1, PKC, cytoskeletal protein actin, proteosome component and HSC70[51]. In addition to phosphorylation, the different spots of vimentin seen on the 2-D gel may be due to association with other molecules, such as protein kinase C, integrin or other associated proteins in signal transduction pathways. Changes in vimentin expression levels are also generated by stress, such as heat shock and oxidative stress[52]. Cleavage of vimentin precedes the recognition of cytoskeletal filaments in apoptosis and programmed cell death mechanisms. Downregulation of vimentin expression inhibits migration and invasion of colon and breast cancer cell lines[53]. According to our Western blot analysis, down-regulated vimentin may suppress tumorigenesis and regulate apoptosis in kojic acid-treated A375 melanoma cells. Moreover, the presence of more than one protein band recognized by the monoclonal antibody of vimentin may be caused by post-translational modifications. Another protein that leads to tumor suppression is pyruvate kinase isoenzyme (KPYM). It is a key sensor for energy consumption and regulation of the glycolytic pathway, in which KPYM links energy-rich metabolites from the flow of glucose carbons to nucleic acid[54]. In yeast cells, overexpression of pyruvate kinases leads to an inhibition of cell proliferation by depletion of glycolytic phosphometabolites. Likewise, pyruvate kinases in eukaryotic cells

are involved in such fundamental processes as cell proliferation, tumor formation and apoptosis[55]. Energy used by cancer cells is preferentially produced from glycolysis, in which p53 was found to modulate the balance between the utilization of the respiratory and glycolytic pathways[56]. According to our results from 2-D PAGE and Western blot data, KPYM upregulated in kojic acid-treated A375 melanoma 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 melanoma cells. Most of these proteins were key factors involved in a wide variety of cellular processes, including cell proliferation, regulation of gene expression, signaling, and chromatin and cytoskeleton organization. Interestingly, our proposed interaction network reveals proteins associated with the regulation of apoptosis, which may lead to suppression of the melanogenesis and tumorigenesis of cancer cells. Moreover, these proteins may be useful biomarkers for use in diagnostic and therapeutic applications of skin cancer. Further functional studies of these proteins may lead to better understanding of the pathogenic mechanisms and cellular response to kojic acid treatment.

According to our investigation of the toxicogenomic effects of arbutin on the gene expression profile of A375 melanoma cells using microarray[26], there was no growth inhibition or morphological change of A375 melanoma cells after 72 h in the presence of 8 μg/ml arbutin, which is a mild arbutin concentration and safe for human skin. The gene expression data showed some tumor suppressor genes as biomarkers in A375 melanoma cells. Although one gene makes one protein, post-translational modifications of proteins can lead to changes in biological and physiological functions that may not result from gene modification. To examine the effects of arbutin on protein expression levels in A375 melanoma cells, the same concentration of arbutin (8 μg/ml) at different treatment times of 24, 48 and 72 h was used. Among the differentially expressed proteins validated by RT-qPCR, VIME, 14-3-3G and VDAC-1 were found to interact with Raf-1. VIME, an IF protein, is the major cytoskeleton component of developing cancer cells. VIME is phosphorylated by Raf-1-associated vimentin kinase, resulting in the regulation of the vimentin filament structure[57]. VIME is also a target for phosphorylation by PKC, which is involved in many signal transduction pathways including the regulation of organelle movement in melanophores[58], cytoskeletal function and programmed cell death (apoptosis)[59]. In carcinoma cell lines, downregulation of VIME expression resulted in impaired migration and adhesion[60]. Likewise, our RT-qPCR results show that VIME is downregulated in arbutin-treated A375 melanoma cells, suggesting an effect on the IF network that ultimately leads to impaired migration and adhesion of A375 melanoma cells. Because VIME is involved in the regulation of stress response proteins[61], the heat shock protein ENPL, also found to be downregulated in A375 melanoma cells in this study, may be involved in VIME regulation. ENPL is a member of the heat shock protein HSP90 complex found in the melanosome. It is well known that heat shock proteins are overexpressed in a wide range of human cancers and are implicated in tumor cell proliferation, differentiation and recognition by the immune system[62]. Therefore, the ENPL downregulated in arbutin-treated A375 melanoma cells may suppress tumor progression and metastasis and cause the decreased immune response.

ENPL was also found to be associated with other types of heat shock proteins and interacts with p53 and 14-3-3 proteins, both of which play important roles in apoptosis. 14-3-3 proteins act as control points for many cellular processes and therefore play significant roles in cell-fate determination and in several apoptotic pathways in animals[63]. 14-3-3 proteins

have been reported to have important interactions with other regulatory proteins. They display important anti-apoptotic characteristics by inhibiting the pro-apoptotic Bcl-2-antagonist of cell death (BAD) and the transcription factor FKHRL-1. Major roles ascribed to the mammalian 14-3-3 proteins include activation of tyrosine and tryptophan hydroxylases, regulation of PKC, exocytosis, especially in mediating interactions between protein kinases, and other signal transduction proteins[64]. 14-3-3 was found to activate the Ras-Raf mitogenic pathway and can elicit a physiologically significant activation of Raf-1 in mammalian cells[65]. Because 14-3-3 proteins are involved in a great number of interactions, the effects of knocking out or overexpressing specific 14-3-3 genes or 14-3-3 target genes will likely have effects on other 14-3-3-regulated cellular processes. Studies have found that 14-3-3 proteins activate p53 function *in vivo*; thus, the up-regulation of 14-3-3G protein expression in arbutin-treated A375 melanoma cells implies that arbutin can stimulate changes in signal transduction pathways by stabilizing or increasing the expression of 14-3-3[66]. The increase in protein expression might involve a critical response of cell proliferation, differentiation and apoptosis of arbutin-treated A375 melanoma cells. The change in the expression of 14-3-3 might alter the biological activities of p53, RAF-1, CLIC1, ENPL, Bcl2 and kinesin-like proteins. Interestingly, the partial functions of ENPL and VDAC-1 on ion channel activity and ion sequestering ability are involved in energy metabolism, which is important for survival. The cellular location of these proteins at the cell membrane and mitochondria implies that the effect of arbutin on membrane and mitochondria may cause changes in cellular physiology and metabolic events. VDAC-1, a major mitochondrial outer membrane transporter, is a component of the permeability transition (PT) and plays an important role in apoptosis by participating in the release of intermembrane space proteins, including cytochrome c, and by its involvement in Ca^{2+} signaling[67]. VDAC-1 has also been found to be associated with the Bax/Bak and Bcl-2 families of proteins, which are essential regulators of cell death and exert their primary pro- or anti-apoptotic roles at the mitochondrial outer membrane. Therefore, the upregulation of VDAC-1 expression in arbutin-treated A375 melanoma cells may play a crucial role in mitochondria-mediated apoptosis, mitochondrial membrane permeability transition and intracellular Ca^{2+} transport. Alternatively, p53 tumor suppressor proteins have been reported to have additional roles in the regulation of glycolysis.

In this study, four proteins identified as glycolytic enzymes, G3P, ENOA, G6PD and MDHM, were found to be involved in glucose metabolism, but only ENOA was validated by RT-PCR. The low expression of ENOA in arbutin-treated A375 melanoma cells may be implicated in metastasis and may also change the associated signaling pathways that modulate cellular metabolism. In addition to regulating glycolysis in cancer cells, p53 also helps to regulate both apoptosis and intracellular reactive oxygen species (ROS) levels. Additionally, peroxiredoxin-1 (PROX1) is one of the antioxidant enzymes and is involved in cellular proliferation and differentiation. The ability of PROX1 to enhance cell survival is traditionally attributed to its capacity to remove ROS[68]. Indeed, lower PROX1 activity increased levels of ROS and induced p53 expression. Therefore, the level of p53 is a major determinant of the effect of decreased PROX1 expression on tumor growth and the response of cells to treatment. This agrees with our results showing up-regulated p53 and down-regulated PROX1 in arbutin-treated A375 melanoma cells. In addition to energy metabolism, arbutin may also affect nucleotide metabolism because ribonucleoproteins (HNRH1, ROA2, ROA1), ribonuclease inhibitor (RINI) and inosine-5'-monophosphate dehydrogenase (IMDH2) were affected by arbutin treatment. IMDH2 is a regulatory enzyme of guanine

nucleotide biosynthesis and is also strategically positioned in the metabolic pathway of thiopurines. Increased mRNA expression levels and enzymatic activity of IMPDH2 have been observed in rapidly proliferating tumor cells. IMDH2 has been established as an anticancer target. Therefore, downregulation of IMDH2 may suppress nucleotide biosynthesis, cell proliferation and malignancy of A375 melanoma cells. According to our proteomic results, many identified proteins, including heat shock proteins, glucose-regulated proteins and other proteins, were found to be associated with p53, which is a major tumor-suppressor protein and plays significant roles in many biological processes in cancer development. Moreover, p53 has biological roles in pigmentation. Tumor suppressor p53 could down- and upregulate tissue-specific expression of the tyrosinase gene in human melanoma cell lines. Therefore, arbutin has potent effects on both protein and gene expression and leads to the suppression of melanogenesis and tumorigenesis of A375 melanoma cells. However, the correlation of protein and gene expression with biological function will be further studied to better understand the effect of arbutin on the biology of A375 malignant melanoma cancer cells. In summary, we identified differentially expressed proteins in arbutin-treated A375 melanoma cells. Most of these proteins were key players in a wide variety of cellular processes, including cell proliferation, regulation of protein expression and signaling pathways.

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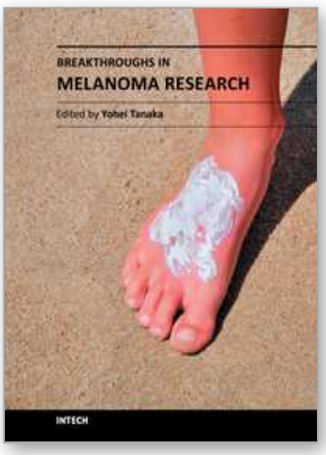

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Melanoma is considered to be one of the most aggressive forms of skin neoplasms. Despite aggressive researches towards finding treatments, no effective therapy exists to inhibit the metastatic spread of malignant melanoma. The 5-year survival rate of metastatic melanoma is still significantly low, and there has been an earnest need to develop more effective therapies with greater anti-melanoma activity. Through the accomplishment of over 100 distinguished and respected researchers from 19 different countries, this book covers a wide range of aspects from various standpoints and issues related to melanoma. These include the biology of melanoma, pigmentations, pathways, receptors and diagnosis, and the latest treatments and therapies to make potential new therapies. Not only will this be beneficial for readers, but it will also contribute to scientists making further breakthroughs in melanoma research.

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