



## Proteomics analysis of A375 human malignant melanoma cells in response to arbutin treatment

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### ABSTRACT

Although the toxicogenomics of A375 human malignant melanoma cells treated with arbutin have been elucidated using DNA microarray, the proteomics of the cellular response to this compound are still poorly understood. In this study, we performed proteomic analyses to investigate the anticancer effect of arbutin on the protein expression profile in A375 cells. After treatment with arbutin (8 µg/ml) for 24, 48 and 72 h, the proteomic profiles of control and arbutin-treated A375 cells were compared, and 26 differentially expressed proteins (7 upregulated and 19 downregulated proteins) were identified by MALDI-Q-TOF MS and MS/MS. Among these proteins, 13 isoforms of six identical proteins were observed. Bioinformatic tools were used to search for protein function and to predict protein interactions. The interaction network of 14 differentially expressed proteins was found to be correlated with the downstream regulation of p53 tumor suppressor and cell apoptosis. In addition, three upregulated proteins (14-3-3G, VDAC-1 and p53) and five downregulated proteins (ENPL, ENOA, IMDH2, PRDX1 and VIME) in arbutin-treated A375 cells were validated by RT-PCR analysis. These proteins were found to play important roles in the suppression of cancer development.

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### 1. Introduction

Standard topical treatments for hyperpigmentation disorders, such as melasma and post-inflammatory hyperpigmentation, and for some dermatological disorders include hydroquinones, retinoids and tyrosinase inhibitors. Tyrosinase inhibitors are becoming increasingly important in the cosmetics and medicinal industries due to their preventive effects on pigmentation disorders. A number of tyrosinase inhibitors have been reported from both natural and synthetic sources, but only a few of these are used as skin-whitening agents, primarily due to various safety concerns. Among the skin-whitening agents, arbutin (hydroquinone-O-β-D-glucopyranoside) is a glycosylated hydroquinone found at high concentrations in certain plants and is widely used as an ingredient in skin care products [1]. Arbutin is effective in the topical treatment of various cutaneous hyperpigmentations. The depigmenting mechanism of arbutin in humans involves the inhibition of melanosomal tyrosinase activity, rather than the suppression of the expression or synthesis of the enzyme [2–5].

Arbutin concentrations in the range of 0.5–8 mM increase the pigmentation of the cultured melanocytes [6]. Arbutin has not been reported to induce gene mutations in the absence of an activating system, although it is strongly mutagenic in the presence of cytosolic fractions from human intestinal bacteria *E. ramulus* and *B. distasonis* [7]. In our previous study, the effect of arbutin on gene expression in human malignant melanoma cells was investigated using microarray. The microarray results indicated that, at safe levels, arbutin can induce a change in gene expression. There were 324 significantly differentially expressed genes (88 upregulated genes and 236 downregulated genes). The correlation of differentially expressed genes with signaling pathways of malignant melanogenesis and tumorigenesis was built by combining the databases of pathways. The significant arbutin-responsive genes, AKT1, FGFR3 and LRP6, are correlated with the AKT, RAS, MAPK and WNT signaling pathways [8]. In addition to having a relationship with the tyrosinase enzyme, their biological activities are also associated with cell proliferation, differentiation and apoptosis [9,10]. These genes play key roles in the regulation of malignant melanogenesis of arbutin-treated A375 cells.

From the microarray results, it was suggested that arbutin has a potential role as an antitumor agent. Although the effects of arbutin on gene expression had previously been determined by microarray, the

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biological effects, functional mechanism, and tumorigenic potential on protein expression levels of malignant tumorigenesis have never been reported. In this study, we used 2-D PAGE to investigate the biological effects of arbutin on the protein expression profile of A375 human malignant melanoma cells and on the melanocytic tumorigenesis and other related side effects of cancer therapy. In addition, we used the bioinformatics tools for searching protein ontology, classification and cellular response in order to gain a better understanding of the regulatory mechanism of arbutin on the protein expression levels of human skin cancer cells.

## 2. Materials and methods

### 2.1. Test substances and chemicals

The human malignant melanoma cell line A375 (CRL-1619) was obtained from ATCC (Rockville, MD, USA). Fetal bovine serum (FBS), sodium pyruvate, antibiotic-antimycotic and TRIzol reagent were purchased from Invitrogen (Carlsbad, CA, USA). Dulbecco's Modified Eagle's Medium (DMEM) and Trypsin-EDTA were purchased from Atlanta Biologicals (Norcross, GA, USA). Arbutin and sodium bicarbonate were purchased from Sigma (St. Louis, MO, USA). IPGphor, immobiline drystrips and carrier ampholytes were purchased from GE Healthcare (Uppsala, Sweden). Urea and CHAPS were purchased from J.T. Baker (Phillipsburg, NJ, USA). Iodoacetamide was purchased from Fluka (Buchs, Switzerland). Dithioerythritol (DTE) was purchased from AppliChem (Darmstadt, Germany). Sypro<sup>®</sup> Ruby was purchased from Molecular Probes (Eugene, OR, USA). Deionized water was prepared with a tandem Milli-Q system (Millipore, Bedford, MA, USA) and used for the preparation of all buffers.

### 2.2. Cell culture and treatment

A375 human malignant melanoma cells were cultured in DMEM medium supplemented with 10% FBS, 1.5 g/ml sodium bicarbonate, 1 mM sodium pyruvate and 1% antibiotic-antimycotic in a humidified incubator with 5% CO<sub>2</sub> at 37 °C. During subculture, the medium was replaced every 2 days. To perform cell attachment, A375 cells were seeded at 1.5 × 10<sup>6</sup> cell/ml in a 110 mm Petri dish cell culture plate (NUNC<sup>™</sup>, Roskilde, Denmark) in 9.0 ml of culture media overnight. The cells were then cultured either alone or in the presence of arbutin at a concentration of 8 µg/ml for 24, 48 and 72 h. The cells were harvested by treatment with trypsin-EDTA, washed two times with sterile PBS and stored at 4 °C until further use.

### 2.3. Two-dimensional electrophoresis (2-D PAGE)

A375 cells were resuspended in 350 µl 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 µg) were applied onto IPG strips (18 cm, pH 3–10NL). Rehydration was performed with an IPGphor IEF system (GE Healthcare) at 20 °C and 30 V for 12 h. Subsequently, IEF was conducted at 8000 V for 70 kVh. After IEF, the IPG strips were equilibrated with equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 2% SDS, 30% glycerol, 2% DTE and a trace of bromophenol blue) for 15 min and then in another similar buffer, where DTE was replaced with 2.5% iodoacetamide (IAA), for a further 15 min. The IPG strips were transferred onto a 12.5% homogeneous polyacrylamide gel (18 × 18 cm), and the second dimensional separation was performed in a Protean II xi Cell at 45 mA per gel at 8 °C until the bromophenol blue dye front reached the bottom of the gel. Proteins were visualized with Sypro<sup>®</sup> Ruby dye. The 2-D gel images were scanned using a Typhoon laser scanner (GE Healthcare, Uppsala, Sweden) and exported to the image analysis software program using PDQuest package software version 7.1.1 (Bio-Rad, Hercules, CA, USA).

### 2.4. In-gel tryptic digestion

Differentially expressed spots were manually excised from the gels and transferred to 500 µl siliconized eppendorf tubes. The gel pieces were washed twice with 200 µl of 50% ACN/25 mM ammonium bicarbonate buffer, pH 8.0, for 15 min each. The gel pieces were then washed once with 200 µl of 100% ACN and dried in a SpeedVac concentrator. Dried gel pieces were swollen in 20 µl of 25 mM ammonium bicarbonate containing 5 ng of sequencing grade modified trypsin (Promega, Madison, WI). Gel pieces were incubated at 37 °C for at least 16 h. Peptides were subsequently extracted three times with 50 µl of 50% ACN/1% TFA, and then the extracted solutions were combined and dried in a SpeedVac concentrator. The peptide pellets were resuspended in 4 µl of 20% ACN/0.1% TFA for MS analysis.

### 2.5. MALDI-TOF MS and MS/MS analysis

The trypsinized samples were premixed in a 1:1 ratio with the matrix solution, containing 5 mg/ml α-cyano-4-hydroxy-cinnamic acid (CHCA) in 50% ACN, 0.1% v/v TFA and 2% w/v ammonium citrate, and spotted onto the 96-well MALDI target plate. The samples were analyzed by the Q-TOF Ultima<sup>™</sup> MALDI instrument (M@LDI<sup>™</sup>; Micromass, Manchester, UK), which was fully automated with a predefined probe motion pattern and peak intensity threshold for switching over from MS survey scanning to MS/MS and from one MS/MS to another. Within each sample well, parent ions that met the predefined criteria (any peak within the m/z 800–3000 range with an intensity above 10 count ± include/exclude list) were selected for CID MS/MS using argon as the collision gas and a mass dependent ± 5 V rolling collision energy until the end of the probe pattern was reached. The MS and MS/MS ion data were extracted and output as searchable .txt and .pkl files, respectively, for independent searches using the MASCOT search engine (<http://www.matrixscience.com>), assuming that peptides were monoisotopic, 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 was performed using the Swiss-Prot human protein database.

### 2.6. RT-PCR

After A375 cells treated with arbutin at 24, 48 and 72 h were collected, total RNA was extracted by a modified method using TRIzol reagent combined with RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The total RNA was quantified by a NanoDrop<sup>®</sup> ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). A mixture of oligo dT, DTT, dNTP, reaction buffer and reverse transcriptase was added to RNA samples. RNA was then converted to cDNA by incubating at 42 °C for 50 min. The cDNA were stored at –20 °C until further use. The sequences of primers used were: (1) 14-3-3G: forward 5'-CGAGCAACTGGTGCAGAAA-3' and reverse 5'-TTCTCATTGCCGTCTGCAGAT-3'; (2) VDAC-1: forward 5'-AGGAATGACGGGACAG AGTTT-3' and reverse 5'-AGATACCCACCGAGAAGCAG-3'; (3) PRDX1: forward 5'-TGTTGTCGGTGGTTAGTTTCT-3' and reverse 5'-TGCCTATCACTGAAAGCAA-3'; (4) ENPL: 5'-ATGGAGATCCTGGAATAGGCA-3' and reverse 5'-GCTTTTC-TCCAATCAAATGGC-3'; (5) IMDH2: forward 5'-CAACACTCATGCCAGGACATT-3' and reverse 5'-TATGGAGCCACCTTCCAC-3'; (6) ENOA: forward 5'-TTTTCTCGCCTCATTCCA-3' and reverse 5'-ATGGGTCACTGAGGCTTTTT-3'; (7) p53: forward 5'-TGAATGAGGCCTTGAATTAAG-3' and reverse 5'-TGGAGTGAGCCCTGCTGTCT-3'; (8) VIME: forward 5'-TCTGGTTGATACCACTCAA-3' and reverse 5'-AGGCACITGAAAGCTGTTTCT-3'. PCR was carried out with a Pxr2 Thermal Cycler (Thermo Fisher Scientific, CA, USA). The PCR reaction began with heating at 94 °C for 5 min, followed by 25 cycles of

denaturation at 94 °C for 1 min, annealing at 52–55 °C for 45 s, extension at 72 °C for 1 min, and concluded with an incubation step at 72 °C for 7 min. The PCR products were analyzed on a 1.5% agarose gels. The agarose gels were stained with ethidium bromide and photographed under UV illumination. The RT-PCR of the encoded genes of differentially expressed proteins was performed in triplicate. ImageMaster software was used to detect the band intensity of all PCR products. The fold expression of the target gene relative to  $\beta$ -actin was calculated for each sample by the formula: expression ratio =  $\Delta$ sample –  $\Delta$ control;  $\Delta$ sample = average band intensity of arbutin-treated sample – average band intensity of  $\beta$ -actin;  $\Delta$ control = average band intensity of control – average band intensity of  $\beta$ -actin.

### 2.7. Statistical analysis

Three triplicate experiments were done for the cell treatment and subsequent 2-D PAGE analysis of the control and arbutin-treated (24, 48 and 72 h) cells. Each group of 2-D gel images was statistically analyzed by image analysis software separately, and the change in protein expression or regulation was determined by dividing the mean spot density of a spot of interest from the arbutin-treated group divided by the mean spot density for the same spot from the control group. Data are expressed as mean value and standard derivation (SD). Differentially expressed protein spots that were found in all sets of experiments were selected for protein identification.

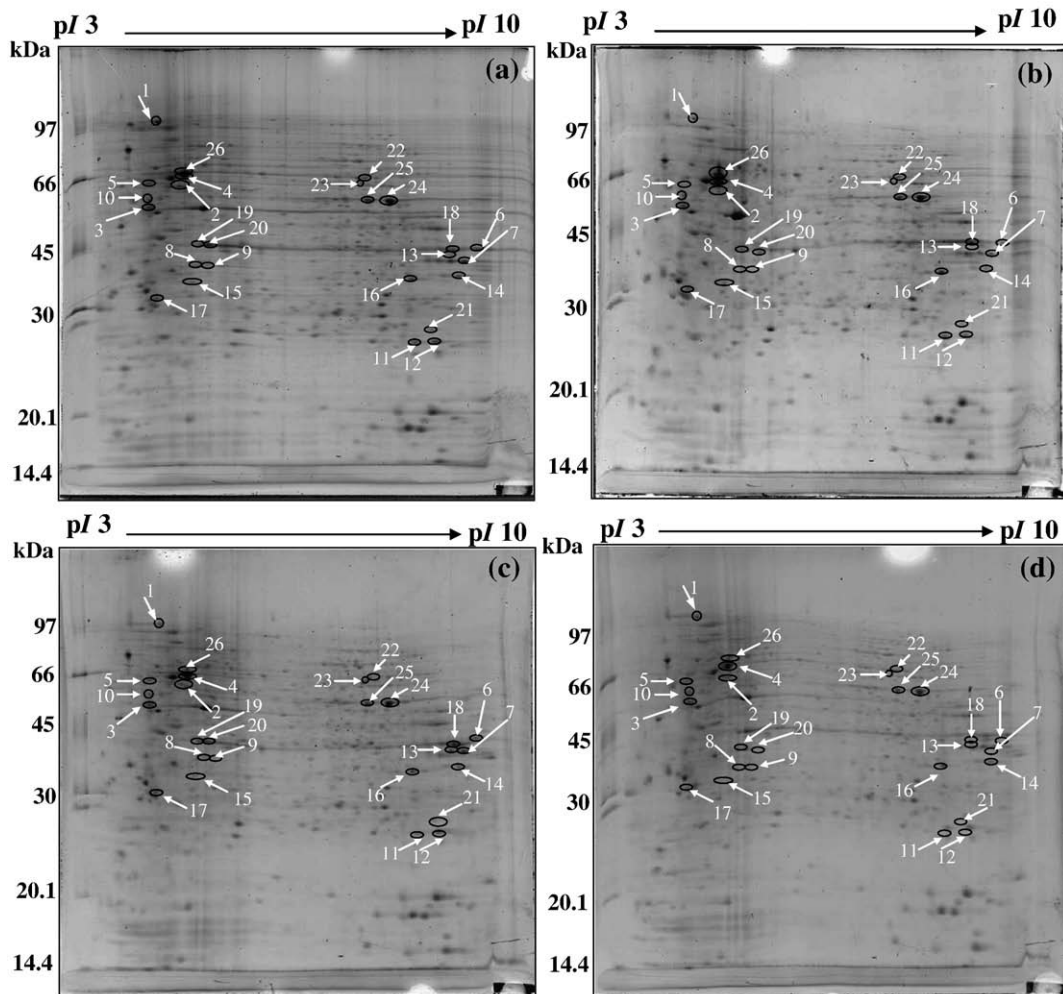
### 2.8. Bioinformatics tools for protein searches

To search protein ontology, we used a combination of databases to gain information on protein annotation, subcellular location, family and superfamily classification, similar proteins, proteins encoding genes, molecular function, biochemically functionally related proteins, and references. Protein searches followed this sequential order of databases: NCBI (<http://www.ncbi.nlm.nih.gov>), Swiss-Prot/TrEMBL (<http://www.expasy.ch/sprot>) and Proteome (<http://www.proteome.com/databases/HumanPD/reports>). In addition, the functional protein association networks or protein interactions were searched by the STRING database (<http://string.embl.de/>). The combination of pathway databases of BioCarta (<http://www.biocarta.com>) and KEGG (<http://www.genome.ad.jp/kegg/pathways.html>) with the NCBI (PubMed) literature (<http://www.ncbi.nlm.nih.gov/PubMed>) was also used to search the correlated regulatory pathways of cellular responses in A375 cells. Protein–protein interaction scores were obtained from the STRING database.

## 3. Results

### 3.1. 2-D PAGE of A375 cells in response to arbutin treatment

The 2-D gel images of A375 cells without treatment (control) and with arbutin treatment at 24, 48 and 72 h are shown in Fig. 1. Most of



**Fig. 1.** 2-D PAGE images of A375 human malignant melanoma cells: (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 an IPG strip pH 3–10 NL. The pointed arrows indicate the identified protein spots in this study.

the proteins were distributed within the molecular weight range from 25 to 97 kDa, and the differentially expressed protein spots were distributed between acidic and basic regions. Using PDQuest image analysis software, the total number of protein spots from the control and treatment groups was about 540 spots with no significant difference among the groups. Among these protein spots, there were 40 differentially expressed protein spots, containing 10 upregulated protein spots, in which the threshold of change in expression level was 1.0 fold, and 40 downregulated protein spots, in which the threshold of change in expression level was 0.9 fold.

### 3.2. Protein 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. A list of identified proteins, Mowse score, apparent and theoretical MW and pI, number of PMF and MS/MS matched sequences, coverage, regulation or change in expression level and standard deviation value are shown in Table 1. The PMF and MS/MS matched peptide sequences of 26 identified proteins are also shown in Table S1. Among these proteins, there were five upregulated and 15 downregulated proteins (including their isoforms).

Most of the identified proteins were downregulated in arbutin-treated A375 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 peroxiredoxins-1, three glyceraldehyde-3-phosphate dehydrogenases and two alpha-enolases, which account for about 50% of all of the identified proteins and might be caused by post-translational modifications.

The differentially expressed proteins in arbutin-treated A375 cells are classified according to cellular component, molecular function and biological process and their protein interaction partners are predicted in Table 2. The identified proteins were distributed to many locations in the cellular component, have diverse functions, and were found to play important roles in many biological processes, especially in cancer biology, such as in apoptosis, regulation of angiogenesis, regulation of cell proliferation, regulation of 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 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 (9 spots including their isoforms, 34.6% of

**Table 1**  
Protein identification of differentially expressed proteins in arbutin-treated A375 cells at 72 h 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	Fold-change	SD
			Apparent	Theoretical						
1	Endoplasmic precursor (Heat shock protein 90 kDa beta member 1) (ENPL)	P14625	98.0, 4.10	92.4, 4.76	MS, MS/MS	87, 159	24, 4	14, 3	-2.50	0.050
2	Protein disulfide-isomerase A6 precursor (PDIA6)	Q15084	64.50, 4.50	48.09, 4.95	MS/MS	-, 54	-, 2	-, 1	-1.00	0.055
3	Vimentin (VIME)	P08670	57.50, 4.00	53.62, 5.06	MS, MS/MS	143, 160	51, 12	17, 4	-2.00	0.125
4	Vimentin (VIME)	P08670	70.80, 4.50	53.62, 5.06	MS, MS/MS	156, 126	47, 9	17, 3	-1.50	0.017
5	Heterogeneous nuclear ribonucleoprotein H (HNRH1)	P31943	65.6, 4.00	49.20, 5.89	MS, MS/MS	41, 51	28, 4	7, 1	-1.20	0.125
6	Heterogeneous nuclear ribonucleoproteins A2/B1 (ROA2)	P22626	46.00, 8.60	37.41, 8.97	MS, -	123, -	48, -	14, -	-0.95	0.006
7	Heterogeneous nuclear ribonucleoproteins A2/B1 (ROA2)	P22626	43.10, 8.30	37.41, 8.97	MS, MS/MS	137, 114	49, 8	15, 2	-0.95	0.003
8	Heterogeneous nuclear ribonucleoprotein A1 (ROA1)	P09651	42.00, 4.70	38.82, 9.26	MS, MS/MS	72, 191	31, 14	9, 4	-2.45	0.011
9	Heterogeneous nuclear ribonucleoprotein A1 (ROA1)	P09651	42.00, 4.90	38.82, 9.26	MS, MS/MS	106, 191	36, 14	12, 1	-2.50	0.017
10	Ribonuclease inhibitor (RINI)	P13489	60.00, 3.95	49.94, 4.71	MS, MS/MS	50, 47	27, 2	9, 1	-2.45	0.013
11	Peroxiredoxin-1 (PRDX1)	Q06830	27.03, 6.90	22.10, 8.27	MS, MS/MS	63, 117	42, 15	7, 2	-2.00	0.054
12	Peroxiredoxin-1 (PRDX1)	Q06830	27.03, 7.40	22.10, 8.27	MS, MS/MS	75, 137	47, 15	8, 2	-2.10	0.111
13	Malate dehydrogenase, mitochondrial precursor (MDHM)	P40926	44.50, 8.80	35.52, 8.92	MS, MS/MS	96, 266	41, 16	11, 2	-2.05	0.008
14	Voltage-dependent anion-selective channel protein 1 (VDAC-1)	P21796	39.60, 8.10	30.85, 8.62	MS, -	55, -	45, -	7, -	+1.35	0.011
15	Chloride intracellular channel protein 1 (CLIC1)	O00299	37.50, 4.65	26.91, 5.09	MS, MS/MS	58, -	39, -	7, -	+1.30	0.013
16	Guanine nucleotide-binding protein subunit beta 2-like (GBLP)	P63244	38.50, 6.85	35.05, 7.60	MS, MS/MS	85, 49	42, 4	10, 1	+2.20	0.110
17	14-3-3 protein gamma (1433G)	P61981	34.00, 4.10	28.28, 4.80	-, MS/MS	-, 46	-, 8	-, 1	+1.50	0.013
18	Glyceraldehyde-3-phosphate dehydrogenase (G3P)	P04406	46.00, 8.75	36.03, 8.57	MS, MS/MS	89, 46	39, 4	11, 1	-1.00	0.085
19	Glyceraldehyde-3-phosphate dehydrogenase (G3P)	P04406	46.50, 4.75	36.03, 8.57	MS, -	75, -	37, -	9, -	-1.50	0.013
20	Glyceraldehyde-3-phosphate dehydrogenase (G3P)	P04406	46.50, 4.90	36.03, 8.57	MS, -	77, -	33, -	11, -	-1.55	0.010
21	Proteasome subunit beta type 1 precursor (PSB1)	P20618	28.30, 7.30	26.47, 8.27	MS, MS/MS	54, 54	42, 13	9, 2	-1.55	0.018
22	Inosine-5'-monophosphate dehydrogenase 2 (IMDH2)	P12268	68.58, 6.15	55.77, 6.44	MS, MS/MS	85, 84	30, 3	12, 1	-1.50	0.026
23	Glucose-6-phosphate 1-dehydrogenase (G6PD)	P11413	65.50, 6.10	59.22, 6.39	MS, MS/MS	82, 75	33, 5	12, 2	-1.30	0.012
24	Alpha-enolase (ENOA)	P06733	60.00, 6.50	47.14, 7.01	MS, MS/MS	125, 299	39, 12	16, 3	-0.90	0.019
25	Alpha-enolase (ENOA)	P06733	60.00, 6.20	47.14, 7.01	MS, MS/MS	111, 217	38, 12	14, 3	-0.90	0.015
26	Tubulin alpha-ubiquitous chain (TBAK)	P68361	71.30, 4.50	50.12, 4.94	MS, MS/MS	123, 140	41, 12	16, 3	+1.00	0.081

**Table 2**  
Protein ontology of identified proteins and some predicted interaction partners

Spot no.	Protein name	Cellular component	Molecular function	Biological process	Predicted some interaction partners
1	Heat shock protein 90 kDa beta member 1 (ENPL)	<ul style="list-style-type: none"> <li>Cytosol</li> <li>Melanosome stage 1–4</li> <li>Endoplasmic reticulum lumen</li> <li>Endoplasmic reticulum membrane</li> </ul>	<ul style="list-style-type: none"> <li>Calcium ion binding</li> <li>Low-density lipoprotein receptor binding</li> <li>RNA binding</li> <li>Virion binding</li> </ul>	<ul style="list-style-type: none"> <li>Anti-apoptosis</li> <li>Protein transport</li> <li>Response to hypoxia</li> <li>Sequestering of calcium ion</li> </ul>	<ul style="list-style-type: none"> <li>Grp75</li> <li>Grp78</li> <li>PDI A4</li> <li>Receptor tyrosinase-protein kinase erbB-2</li> <li>p53</li> <li>ERp31</li> <li>GRP78</li> </ul>
2	Protein disulfide-isomerase A6 precursor (PDIA6)	<ul style="list-style-type: none"> <li>Endoplasmic reticulum</li> <li>Melanosome stage 1–4</li> <li>ER–Golgi intermediate compartment</li> </ul>	<ul style="list-style-type: none"> <li>Protein binding</li> <li>Protein disulfide isomerase activity</li> </ul>	<ul style="list-style-type: none"> <li>Protein folding</li> </ul>	<ul style="list-style-type: none"> <li>RAF-1</li> <li>Proteasome subunit alpha type1</li> <li>Protein kinase N1</li> <li>p53</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>NHP2-like protein1</li> <li>ATP-dependent RNA helicase A</li> <li>Polyadenylate binding protein 2</li> <li>Splicing factors</li> <li>Nuclear DNA helicase II</li> <li>Nuclease sensitive element binding protein1</li> <li>Element binding protein1</li> <li>TNF-alpha</li> <li>NF-kappa-B essential modulator</li> <li>Receptor-interacting serine/threonine-protein kinase 3</li> <li>Interleukin-4</li> <li>Interferon regulatory factor 3</li> <li>p53-regulated proteins PA26</li> </ul>
3,4	Vimentin (VIME)	<ul style="list-style-type: none"> <li>Cytoplasm</li> <li>Intermediate filament</li> </ul>	<ul style="list-style-type: none"> <li>Protein binding</li> <li>Structural constituent of cytoskeleton</li> </ul>	<ul style="list-style-type: none"> <li>Cell motility</li> </ul>	<ul style="list-style-type: none"> <li>RAF-1</li> <li>Proteasome subunit alpha type1</li> <li>Protein kinase N1</li> <li>p53</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>NHP2-like protein1</li> <li>ATP-dependent RNA helicase A</li> <li>Polyadenylate binding protein 2</li> <li>Splicing factors</li> <li>Nuclear DNA helicase II</li> <li>Nuclease sensitive element binding protein1</li> <li>Element binding protein1</li> <li>TNF-alpha</li> <li>NF-kappa-B essential modulator</li> <li>Receptor-interacting serine/threonine-protein kinase 3</li> <li>Interleukin-4</li> <li>Interferon regulatory factor 3</li> <li>p53-regulated proteins PA26</li> </ul>
5	Heterogeneous nuclear ribonucleoprotein H (HNRH1)	<ul style="list-style-type: none"> <li>Heterogeneous nuclear ribonucleoprotein complex</li> </ul>	<ul style="list-style-type: none"> <li>Poly(U) binding</li> <li>Protein binding</li> </ul>	<ul style="list-style-type: none"> <li>RNA processing</li> </ul>	<ul style="list-style-type: none"> <li>RAF-1</li> <li>Proteasome subunit alpha type1</li> <li>Protein kinase N1</li> <li>p53</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>NHP2-like protein1</li> <li>ATP-dependent RNA helicase A</li> <li>Polyadenylate binding protein 2</li> <li>Splicing factors</li> <li>Nuclear DNA helicase II</li> <li>Nuclease sensitive element binding protein1</li> <li>Element binding protein1</li> <li>TNF-alpha</li> <li>NF-kappa-B essential modulator</li> <li>Receptor-interacting serine/threonine-protein kinase 3</li> <li>Interleukin-4</li> <li>Interferon regulatory factor 3</li> <li>p53-regulated proteins PA26</li> </ul>
6,7	Heterogeneous nuclear ribonucleoproteins A2/B1 (ROA2)	<ul style="list-style-type: none"> <li>Heterogeneous nuclear ribonucleoprotein complex</li> <li>Spliceosome</li> </ul>	<ul style="list-style-type: none"> <li>Protein binding</li> <li>RNA binding</li> <li>Single-stranded telomeric DNA binding</li> </ul>	<ul style="list-style-type: none"> <li>Nuclear mRNA splicing, via spliceosome</li> <li>RNA transport</li> </ul>	<ul style="list-style-type: none"> <li>RAF-1</li> <li>Proteasome subunit alpha type1</li> <li>Protein kinase N1</li> <li>p53</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>NHP2-like protein1</li> <li>ATP-dependent RNA helicase A</li> <li>Polyadenylate binding protein 2</li> <li>Splicing factors</li> <li>Nuclear DNA helicase II</li> <li>Nuclease sensitive element binding protein1</li> <li>Element binding protein1</li> <li>TNF-alpha</li> <li>NF-kappa-B essential modulator</li> <li>Receptor-interacting serine/threonine-protein kinase 3</li> <li>Interleukin-4</li> <li>Interferon regulatory factor 3</li> <li>p53-regulated proteins PA26</li> </ul>
8,9	Heterogeneous nuclear ribonucleoprotein A1 (ROA1)	<ul style="list-style-type: none"> <li>Cytoplasm</li> <li>Heterogeneous nuclear ribonucleo-protein complex</li> <li>Nucleoplasm</li> <li>Spliceosome</li> </ul>	<ul style="list-style-type: none"> <li>Protein binding</li> <li>Single-stranded DNA binding</li> <li>Single-stranded RNA binding</li> </ul>	<ul style="list-style-type: none"> <li>Nuclear import</li> <li>Nuclear mRNA splicing, via spliceosome</li> <li>RNA export from nucleus</li> </ul>	<ul style="list-style-type: none"> <li>RAF-1</li> <li>Proteasome subunit alpha type1</li> <li>Protein kinase N1</li> <li>p53</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>NHP2-like protein1</li> <li>ATP-dependent RNA helicase A</li> <li>Polyadenylate binding protein 2</li> <li>Splicing factors</li> <li>Nuclear DNA helicase II</li> <li>Nuclease sensitive element binding protein1</li> <li>Element binding protein1</li> <li>TNF-alpha</li> <li>NF-kappa-B essential modulator</li> <li>Receptor-interacting serine/threonine-protein kinase 3</li> <li>Interleukin-4</li> <li>Interferon regulatory factor 3</li> <li>p53-regulated proteins PA26</li> </ul>
10	Ribonuclease inhibitor (RINI)	<ul style="list-style-type: none"> <li>Angiogenin-PRI complex</li> </ul>	<ul style="list-style-type: none"> <li>Protein binding</li> <li>Ribonuclease inhibitor activity</li> </ul>	<ul style="list-style-type: none"> <li>mRNA catabolic process</li> <li>Regulation of angiogenesis</li> </ul>	<ul style="list-style-type: none"> <li>RAF-1</li> <li>Proteasome subunit alpha type1</li> <li>Protein kinase N1</li> <li>p53</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>NHP2-like protein1</li> <li>ATP-dependent RNA helicase A</li> <li>Polyadenylate binding protein 2</li> <li>Splicing factors</li> <li>Nuclear DNA helicase II</li> <li>Nuclease sensitive element binding protein1</li> <li>Element binding protein1</li> <li>TNF-alpha</li> <li>NF-kappa-B essential modulator</li> <li>Receptor-interacting serine/threonine-protein kinase 3</li> <li>Interleukin-4</li> <li>Interferon regulatory factor 3</li> <li>p53-regulated proteins PA26</li> </ul>
11, 12	Peroxiredoxin-1 (Thioredoxin peroxidase 2) (PRDX1)	<ul style="list-style-type: none"> <li>Cytoplasm</li> <li>Melanosome stage 1–4</li> </ul>	<ul style="list-style-type: none"> <li>Redox regulation of the cell</li> <li>Participate in the signaling cascades of growth factors and TNF-<math>\alpha</math> by regulating the intracellular concentrations of H<sub>2</sub>O<sub>2</sub></li> </ul>	<ul style="list-style-type: none"> <li>Cell proliferation</li> <li>Skeletal development</li> </ul>	<ul style="list-style-type: none"> <li>RAF-1</li> <li>Proteasome subunit alpha type1</li> <li>Protein kinase N1</li> <li>p53</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>NHP2-like protein1</li> <li>ATP-dependent RNA helicase A</li> <li>Polyadenylate binding protein 2</li> <li>Splicing factors</li> <li>Nuclear DNA helicase II</li> <li>Nuclease sensitive element binding protein1</li> <li>Element binding protein1</li> <li>TNF-alpha</li> <li>NF-kappa-B essential modulator</li> <li>Receptor-interacting serine/threonine-protein kinase 3</li> <li>Interleukin-4</li> <li>Interferon regulatory factor 3</li> <li>p53-regulated proteins PA26</li> </ul>
13	Malate dehydrogenase (MDHM)	<ul style="list-style-type: none"> <li>Mitochondrion matrix</li> </ul>	<ul style="list-style-type: none"> <li>l-malate dehydrogenase activity</li> </ul>	<ul style="list-style-type: none"> <li>Citric acid cycle</li> <li>Gluconeogenesis</li> </ul>	<ul style="list-style-type: none"> <li>RAF-1</li> <li>Proteasome subunit alpha type1</li> <li>Protein kinase N1</li> <li>p53</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>NHP2-like protein1</li> <li>ATP-dependent RNA helicase A</li> <li>Polyadenylate binding protein 2</li> <li>Splicing factors</li> <li>Nuclear DNA helicase II</li> <li>Nuclease sensitive element binding protein1</li> <li>Element binding protein1</li> <li>TNF-alpha</li> <li>NF-kappa-B essential modulator</li> <li>Receptor-interacting serine/threonine-protein kinase 3</li> <li>Interleukin-4</li> <li>Interferon regulatory factor 3</li> <li>p53-regulated proteins PA26</li> </ul>
14	Voltage-dependent anion-selective channel protein 1 (VDAC-1)	<ul style="list-style-type: none"> <li>Mitochondrial outer membrane</li> <li>Pore complex</li> </ul>	<ul style="list-style-type: none"> <li>Apoptogenic cytochrome c release channel activity</li> <li>Protein binding</li> <li>Voltage-gated anion channel porin activity</li> </ul>	<ul style="list-style-type: none"> <li>Anion transport</li> <li>Apoptotic program</li> </ul>	<ul style="list-style-type: none"> <li>RAF-1</li> <li>Proteasome subunit alpha type1</li> <li>Protein kinase N1</li> <li>p53</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>NHP2-like protein1</li> <li>ATP-dependent RNA helicase A</li> <li>Polyadenylate binding protein 2</li> <li>Splicing factors</li> <li>Nuclear DNA helicase II</li> <li>Nuclease sensitive element binding protein1</li> <li>Element binding protein1</li> <li>TNF-alpha</li> <li>NF-kappa-B essential modulator</li> <li>Receptor-interacting serine/threonine-protein kinase 3</li> <li>Interleukin-4</li> <li>Interferon regulatory factor 3</li> <li>p53-regulated proteins PA26</li> </ul>
15	Chloride intracellular channel protein 1 (CLIC1)	<ul style="list-style-type: none"> <li>Brush border</li> <li>Cytoplasm</li> <li>Membrane fraction</li> <li>Nuclear envelope</li> <li>Soluble fraction</li> </ul>	<ul style="list-style-type: none"> <li>Chloride channel activity</li> <li>Protein binding</li> </ul>	<ul style="list-style-type: none"> <li>Chloride transport</li> <li>Signal transduction</li> </ul>	<ul style="list-style-type: none"> <li>RAF-1</li> <li>Proteasome subunit alpha type1</li> <li>Protein kinase N1</li> <li>p53</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>NHP2-like protein1</li> <li>ATP-dependent RNA helicase A</li> <li>Polyadenylate binding protein 2</li> <li>Splicing factors</li> <li>Nuclear DNA helicase II</li> <li>Nuclease sensitive element binding protein1</li> <li>Element binding protein1</li> <li>TNF-alpha</li> <li>NF-kappa-B essential modulator</li> <li>Receptor-interacting serine/threonine-protein kinase 3</li> <li>Interleukin-4</li> <li>Interferon regulatory factor 3</li> <li>p53-regulated proteins PA26</li> </ul>
16	Guanine nucleotide-binding protein subunit beta 2-like (GBLP)	<ul style="list-style-type: none"> <li>Cytoplasm</li> </ul>	<ul style="list-style-type: none"> <li>Protein kinase C binding</li> </ul>	<ul style="list-style-type: none"> <li>Cell proliferation</li> <li>Cell differentiation</li> <li>Signal transduction</li> <li>Intracellular protein transportation</li> </ul>	<ul style="list-style-type: none"> <li>RAF-1</li> <li>Proteasome subunit alpha type1</li> <li>Protein kinase N1</li> <li>p53</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>NHP2-like protein1</li> <li>ATP-dependent RNA helicase A</li> <li>Polyadenylate binding protein 2</li> <li>Splicing factors</li> <li>Nuclear DNA helicase II</li> <li>Nuclease sensitive element binding protein1</li> <li>Element binding protein1</li> <li>TNF-alpha</li> <li>NF-kappa-B essential modulator</li> <li>Receptor-interacting serine/threonine-protein kinase 3</li> <li>Interleukin-4</li> <li>Interferon regulatory factor 3</li> <li>p53-regulated proteins PA26</li> </ul>
17	14-3-3 protein gamma (Protein kinase C inhibitor protein 1 (1433G))	<ul style="list-style-type: none"> <li>Cytoplasm</li> </ul>	<ul style="list-style-type: none"> <li>Insulin-like growth factor receptor binding</li> <li>Protein kinase C binding</li> <li>Protein kinase C inhibitor activity</li> </ul>	<ul style="list-style-type: none"> <li>Regulation of signal transduction</li> <li>Regulation of apoptosis</li> <li>Cell cycle progression</li> <li>Regulation of DNA replication</li> <li>Regulation of neuron differentiation</li> <li>Regulation of synaptic plasticity</li> <li>Glycolysis</li> <li>Maintenance of cell function</li> <li>Protein regulation and degradation</li> </ul>	<ul style="list-style-type: none"> <li>RAF-1</li> <li>Proteasome subunit alpha type1</li> <li>Protein kinase N1</li> <li>p53</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>NHP2-like protein1</li> <li>ATP-dependent RNA helicase A</li> <li>Polyadenylate binding protein 2</li> <li>Splicing factors</li> <li>Nuclear DNA helicase II</li> <li>Nuclease sensitive element binding protein1</li> <li>Element binding protein1</li> <li>TNF-alpha</li> <li>NF-kappa-B essential modulator</li> <li>Receptor-interacting serine/threonine-protein kinase 3</li> <li>Interleukin-4</li> <li>Interferon regulatory factor 3</li> <li>p53-regulated proteins PA26</li> </ul>
18, 19, 20	Glyceraldehyde-3-phosphate dehydrogenase (G3P)	<ul style="list-style-type: none"> <li>Cytoplasm</li> </ul>	<ul style="list-style-type: none"> <li>Glyceraldehyde-3-phosphate dehydrogenase</li> <li>Protein binding</li> </ul>	<ul style="list-style-type: none"> <li>Maintenance of cell function</li> </ul>	<ul style="list-style-type: none"> <li>RAF-1</li> <li>Proteasome subunit alpha type1</li> <li>Protein kinase N1</li> <li>p53</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>NHP2-like protein1</li> <li>ATP-dependent RNA helicase A</li> <li>Polyadenylate binding protein 2</li> <li>Splicing factors</li> <li>Nuclear DNA helicase II</li> <li>Nuclease sensitive element binding protein1</li> <li>Element binding protein1</li> <li>TNF-alpha</li> <li>NF-kappa-B essential modulator</li> <li>Receptor-interacting serine/threonine-protein kinase 3</li> <li>Interleukin-4</li> <li>Interferon regulatory factor 3</li> <li>p53-regulated proteins PA26</li> </ul>
21	Proteasome subunit beta type 1 precursor (PSB1)	<ul style="list-style-type: none"> <li>Cytoplasm</li> <li>Nucleus</li> <li>Proteasome complex</li> </ul>	<ul style="list-style-type: none"> <li>Proteinase</li> <li>Protein binding</li> </ul>	<ul style="list-style-type: none"> <li>Protein regulation and degradation</li> </ul>	<ul style="list-style-type: none"> <li>RAF-1</li> <li>Proteasome subunit alpha type1</li> <li>Protein kinase N1</li> <li>p53</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>Heterogeneous nuclear ribonucleoproteins</li> <li>Splicing factors</li> <li>NHP2-like protein1</li> <li>ATP-dependent RNA helicase A</li> <li>Polyadenylate binding protein 2</li> <li>Splicing factors</li> <li>Nuclear DNA helicase II</li> <li>Nuclease sensitive element binding protein1</li> <li>Element binding protein1</li> <li>TNF-alpha</li> <li>NF-kappa-B essential modulator</li> <li>Receptor-interacting serine/threonine-protein kinase 3</li> <li>Interleukin-4</li> <li>Interferon regulatory factor 3</li> <li>p53-regulated proteins PA26</li> </ul>

(continued on next page)

**Table 2** (continued)

Spot no.	Protein name	Cellular component	Molecular function	Biological process	Predicted some interaction partners
22	Inosine-5'-monophosphate dehydrogenase 2 (IMDH2)	• Cytoplasm	• IMP dehydrogenase activity • Rate-limiting enzyme in the de novo synthesis of guanine nucleotides • Protein binding	• Involved in the regulation of cell growth • Guanine nucleotides biosynthesis • Growth progression of some tumors	• Inosine-5' monophosphate dehydrogenase 1 • GMP synthase, • GMP reductase 1 and 2 • AMP deaminase 1 and 3
23	Glucose-6-phosphate 1-dehydrogenase (G6PD)	• Cytoplasm	• Glucose-6-phosphate dehydrogenase activity	• Glucose 6-phosphate utilization • Pentose-phosphate shunt	• NF-kappa-B essential modulator • cGMP-dependent protein kinase 1 • Glucose-6-phosphate isomerase
24, 25	Alpha-enolase (ENOA)	• Cytoplasm • Cell membrane • Nucleus	• Phosphopyruvate hydratase activity • Transcription corepressor activity • Transcription factor activity	• Negative regulation of cell growth • Negative regulation of transcription from RNA polymerase II promoter	• Pyruvate kinase isozymes M1/M2 • Myc proto-oncogene protein • Adenylate kinase isoenzyme 2
26	Tubulin alpha-ubiquitous chain (TBAK)	• Cytosol	• Including motility • Maintenance of cell shape • Intracellular trafficking of macromolecules and organelles • Mitosis	–	• Tubulin beta and alpha chains • Kinesin-like protein KIF1A • Actin

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.

### 3.3. Validation by western blotting or RT-PCR

Among the identified proteins, vimentin (VIME), 4-3-3G, peroxiredoxin-1 (PRDX1), heat shock protein 90 kDa beta member 1 (ENPL), inosine-5'-monophosphate dehydrogenase 2 (IMDH2), ENOA, VDAC-1 and p53 were validated by RT-PCR (Fig. 2). The results of protein and gene expression levels of eight differentially expressed proteins were in agreement with the 2-D PAGE expression data. 14-3-3G, VDAC-1 and p53 proteins were upregulated in the arbutin-treated A375 cells, whereas VIME, PRDX1, ENPL, IMDH2 and ENOA were downregulated in the arbutin-treated A375 cells.

### 3.4. Correlation of differentially expressed proteins with tumorigenesis

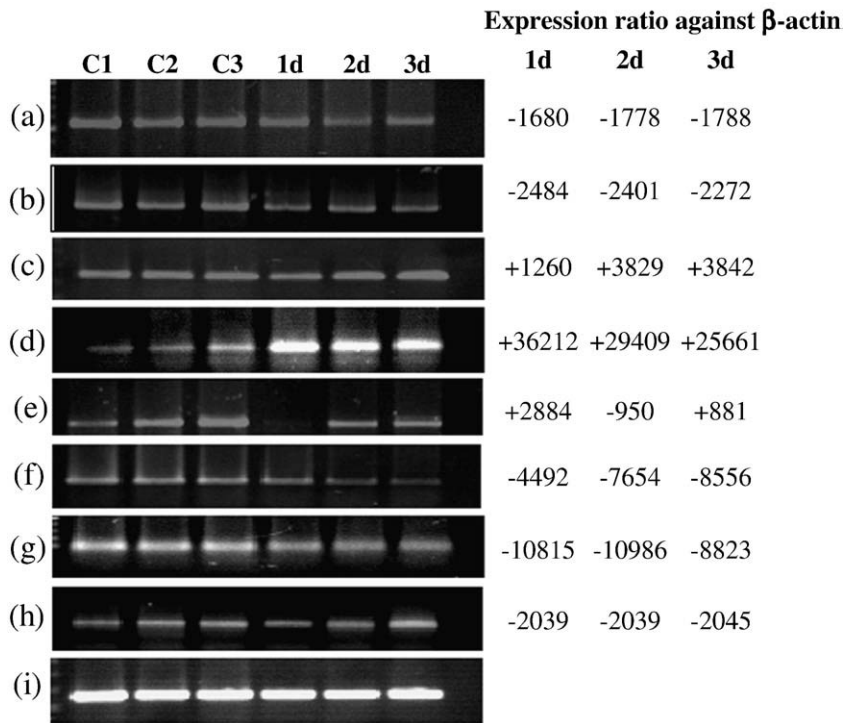
Combining the interaction and pathway databases and literature of STRING, BioCarta, KEGG and PubMed, we built a hypothetical model of a protein interaction network corresponding to the regulation of apoptosis in tumorigenesis wherein 14 differentially expressed proteins in arbutin-treated A375 cells, PDIA6, ENPL, RINI, VDAC-1, CLIC1, PRDX1, VIME, 14-3-3G, PSB1, GBLP, G3P, ENOA, G6PD and MDHM, are correlated (Fig. 3). This network can be divided to four network groups: stress responding proteins, proteins involved in melanogenesis, proteins involved in the regulation of apoptosis and proteins involved in energy metabolism. Among these proteins, there are nine proteins that interacted with protein kinases: ENPL, VIME, RINI, VDAC-1, CLIC1, 14-3-3G, G6PD, GBLP and ENOA; four proteins interacted with p53: ENPL, VIME, PRDX1 and 14-3-3G; two proteins interacted with Bcl2 protein: 14-3-3G and VDAC-1; four proteins were involved in energy metabolism: G3P, ENOA, G6PD and MDHM; and three proteins were involved in ion channels or ion sequestering: VDAC-1, CLIC1 and ENPL. The interaction scores between the proteins were determined using the STRING database (Table S2). However, some of the interactions were not scored since

the interactions had already been reported in the literature. We suggest that arbutin changes the protein expression of A375 cells and may cause some changes in cellular physical and metabolic events that are involved in the regulation of melanogenesis and apoptosis in A375 cells.

## 4. Discussion

According to our previous study that investigated the toxicogenomic effects of arbutin on the gene expression profile of A375 cells using microarray [8], there was no growth inhibition or morphological change of A375 cells after 72 h in the presence of arbutin (8 µg/ml), using a mild arbutin concentration for human skin safety. The gene expression data showed some tumor suppressor genes as biomarkers in A375 cells. Although one gene makes one protein, the post-translational modifications of proteins can lead to changes in biological and physiological functions that may not be caused by gene modification. In order to examine the effects of arbutin on the protein expression levels in A375 cells, the same concentration of arbutin (8 µg/ml), although with different treatment times of 24, 48 and 72 h, was used in this study.

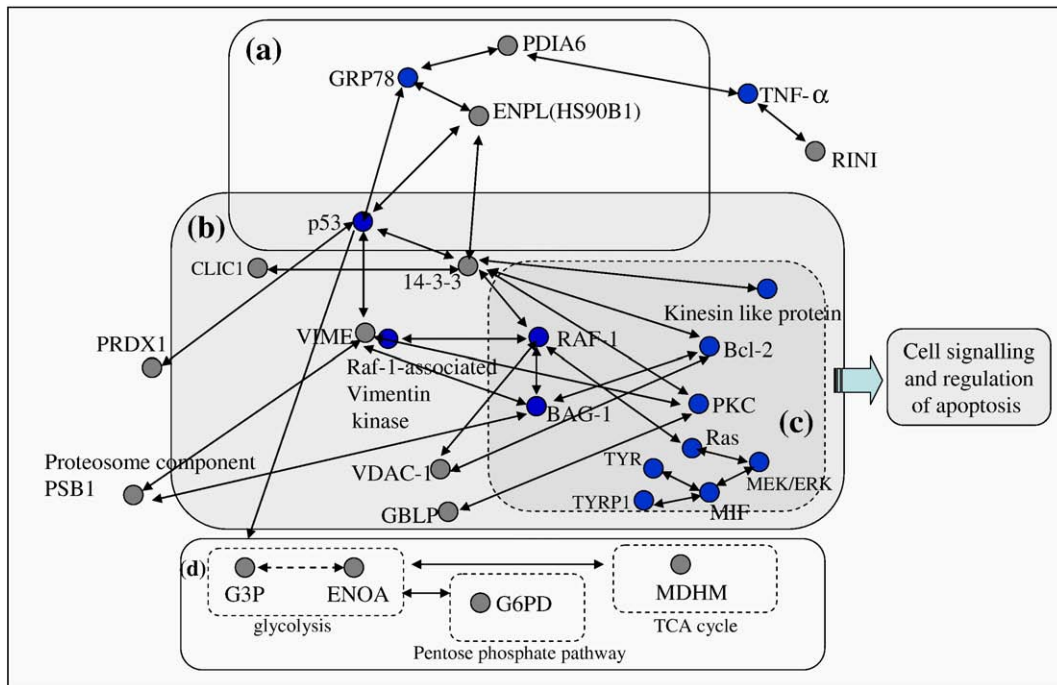
Among the differentially expressed proteins validated by RT-PCR, VIME, 14-3-3G and VDAC-1 were found to interact with Raf-1. VIME, which is one of the intermediate filaments, is the major cytoskeleton component in developing cancer cells. VIME is phosphorylated by Raf-1-associated vimentin kinase, resulting in the regulation of the vimentin filament structure [11]. The protein is also a target for phosphorylation by PKC, which is involved in many signal transduction pathways, including the regulation of organelle movement in melanophores [12–14], regulation of cytoskeletal function and programmed cell death (apoptosis) [15–18]. In carcinoma cell lines, the downregulation of VIME expression resulted in impaired migration and adhesion [19]. Likewise, our RT-PCR results of VIME showed that VIME was downregulated in arbutin-treated A375 cells, suggesting an effect on the intermediate filament network, ultimately leading to impaired migration and adhesion of A375 cells. Since VIME is involved in the regulation of stress response proteins [20], the heat shock protein ENPL, also determined in this study to be downregulated in A375 cells, may be partially involved in VIME regulation. ENPL is a member of the heat shock protein HSP90 that can be 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 [21–24]. Therefore, the downregulated ENPL in



**Fig. 2.** Validations of differentially expressed proteins by RT-PCR analyses. (a) VIME, (b) ENPL, (c) 14-3-3G, (d) VDCA-1, (e) ENOA, (f) PROX1, (g) IMDH2, (h) p53 and (i)  $\beta$ -actin. Labels: c1, c2 and c3 indicate the control group at 24, 48 and 72 h, respectively, and d1, d2 and d3 indicate the treatment groups at 24, 48 and 72 h, respectively.

arbutin-treated A375 cells may suppress the tumor progression and metastasis as well as the decreased immune response. In addition, ENPL was found to be correlated with other types of heat shock proteins and also to interact with p53 and 14-3-3 proteins, which both play important roles in apoptosis.

14-3-3 molecules act as control points for many cellular processes [25,26] and therefore play significant roles in the determination of a cell's fate and in several apoptotic pathways in animals. 14-3-3 proteins have been reported to have important interactions with other regulatory proteins. They display important anti-apoptotic



**Fig. 3.** Proposed interaction network of differentially expressed proteins correlated with the regulation of apoptosis. The interaction network includes the direct and indirect interactions. The gray spots indicate the proteins that were identified in this study, and the blue spots indicate the proteins that were not identified in this study but were found to interact with the identified proteins. Labels: (a) proteins involved in stress, (b) proteins involved in the regulation of cell signaling and apoptosis, (c) proteins involved in the melanogenesis pathway and (d) proteins involved in energy metabolism.

characteristics by inhibiting the pro-apoptotic BAD (Bcl-2-antagonist of cell death) and transcription factor FKHL-1. The major roles ascribed to the mammalian 14-3-3 proteins include activation of tyrosine and tryptophan hydroxylases, regulation of protein kinase C (PKC), exocytosis, especially in mediating interactions between protein kinases and other signal transduction proteins [25]. 14-3-3 was found to activate the Ras-Raf mitogenic pathway. The protein can elicit a physiologically significant activation of Raf-1 in mammalian cells [27]. Since 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. Previous studies found that 14-3-3 proteins activate p53 function *in vivo* [28,29]; thus, the upregulation of 14-3-3G protein expression in arbutin-treated A375 cells may imply that arbutin can stimulate changes within signal transduction pathways by stabilizing or increasing the expression of the protein 14-3-3. The increase in protein expression might involve a critical response of the cell proliferation and differentiation and on apoptosis of the arbutin-treated A375 cells. As shown in Fig. 2, 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 in ion channel activity or ion sequestering ability are involved in energy metabolism, which is important for the cell's survival. Considering the cellular location of these proteins to the cell membrane and mitochondria implies that the effect of arbutin on membrane and mitochondria may cause changes in the cellular physiology and metabolic events. VDAC-1, a major mitochondrial outer membrane transporter, is one of the permeability transition (PT) components [30–32] and plays an important role in apoptosis by participating in the release of intermembrane space proteins, including cytochrome c, and being involved in Ca<sup>2+</sup> signaling [33–38]. VDAC-1 has also been found to be correlated 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 [39–41]. Therefore, the upregulation of VDAC-1 expression in arbutin-treated A375 cells may play a crucial role in the processes of mitochondria-mediated apoptosis, mitochondrial membrane permeability transition and intracellular Ca<sup>2+</sup> transport [42].

On the other hand, p53 tumor suppressor proteins have been reported to have additional roles in the regulation of glycolysis [43]. 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 cells may be implicated in metastasis and also change the correlated signaling pathways that are able to modulate cellular metabolism. In addition to the regulation of glycolysis in cancer cells, p53 also has functions in the regulation of both apoptosis and intracellular reactive oxygen species (ROS) levels [44–47]. Additionally, peroxiredoxin-1 (PROX1) is one of the antioxidant enzymes and is involved in cellular proliferation and differentiation [48]. The cell survival-enhancing function of PROX1 is traditionally attributed to its reactive oxygen species-removing capacity [49]. Indeed, lower PROX1 activity increased reactive oxygen species levels and induced p53 expression [50]. Therefore, the p53 status is a major determinant of the decreased PROX1 expression on tumor growth and the response of cells to treatment. This agrees with our results of upregulated p53 and downregulated PROX1 in arbutin-treated A375 cells.

In addition to energy metabolism, arbutin may also affect nucleotide metabolism since 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 [51]. Increased enzymatic activity of IMPDH2 and its mRNA expression

level have been observed in rapidly proliferating tumor cells [52–55]. The IMDH2 protein has been established as an anticancer target [56]. Therefore, the downregulated IMDH2 may suppress nucleotide biosynthesis, cell proliferation and the malignancy of A375 cells.

According to our proteomic results, many identified proteins, including heat shock proteins, glucose regulated proteins and other proteins, were found to be correlated with p53, which is the major suppressor protein and plays significant roles in many biological processes in cancer development. Moreover, the p53 protein has biological roles in pigmentation. Tumor suppressor p53 could down-regulate the tissue-specific expression of the tyrosinase gene in human melanoma cell lines [57,58]. Therefore, arbutin has potent effects on both protein and gene expression levels and leads to the suppression of the melanogenesis and tumorigenesis of A375 cells. However, the correlation of protein and gene expressions and their biological functions will be further studied for better understanding the biological effect of arbutin on the biology of A375 malignant melanoma cancer cells.

In summary, we identified a number of differentially expressed proteins in arbutin-treated A375 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. Interestingly, our proposed interaction network showed the correlation of some differentially expressed proteins with the regulation of apoptosis, which may lead to the suppression of the melanogenesis and tumorigenesis of cancer cells. However, further functional studies of these proteins may lead to a deeper understanding of the pathogenic mechanisms and cellular responses to arbutin treatment.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbapap.2008.09.023.

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