Immune-associated proteins with potential *in vivo* anti-tumor activities are upregulated in lung cancer cells treated with umbelliprenin: A proteomic approach

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Abstract. Umbelliprenin (Umb), a natural coumarin, has demonstrated anti-tumor activities, both *in vitro* and particularly *in vivo,* in several types of cancer, including lung cancer. The present study aimed to identify molecular targets of Umb using a high-throughput approach. Lung cancer cell lines, QU-DB (large-cell lung carcinoma) and A549 (adenocarcinoma), were treated with Umb. Differentially-expressed proteins were identified using two-dimensional electrophoresis coupled to mass spectrometry. In the QU-DB cells, differential expression of proteins, including downregulation of the tumorigenic protein heat shock protein 90 kDa and upregulation of the potential anti-tumor proteins Nipsnap1 and glycine-tRNA ligase (GRS), suggested that Umb is a strong anti-tumor compound. In the A549 cells, differential expression of proteins indicated possible contradictory effects of Umbregarding tumorigenesis, which included downregulation of the tumorigenic protein cyclophilin and the tumor suppressor MST, and upregulation of stathmin (tumorigenic) and calreticulin. Calreticulun, in addition to GRS in QU-DB cells, stimulates anti-tumor immune responses *in vivo*. To the best of our knowledge, the present study is the first to use a high-throughput approach to identify targets of Umb in cancer. These molecular targets suggested that Umb may exhibit stronger *in vitro* anti-tumor activity against the large-cell carcinoma model than the adenocarcinoma model. Furthermore, it has been reported that Umb exhibits higher cytotoxicity against QU-DB cells than A549 cells *in vitro*, and significant Umb anti-tumor activity against lung cancer *in vivo*, which is consistent with previously published literature. In each cell type, immune-associated molecules were upregulated, indicating that this naturally occurring compound exhibits marked anti-tumor activity *in vivo*. However, further studies that investigate the effect of Umb in different *in vitro* models of cancer are required.

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Introduction

The limited success and considerable side effects of classic chemotherapeutic agents has led to researchers attempting to identify natural anti-tumor compounds with fewer side effects. Umbelliprenin (Umb; $C_{24}H_{30}O_3$; molecular weight, 366), a member of 7-prenyloxycoumarins, is a naturally occurring compound, which is isolated from *Ferula* species, including *Ferula szowitsiana* (1,2). This compound is able to suppress tumorigenesis through its anti-inflammatory, anti-genotoxicity, anti-invasive and lipoxygenase inhibitory activities (3-6). *In vitro*, Umb has been reported to attenuate the proliferation of various cancer cell lines, and is able to prevent/delay cancer in *in vivo* animal models of papilloma and lung cancer (7-9). It hasbeen stated that the anti-tumor activities of Umb are partly due to a prenyl moiety (C7-OH) on the structure of the umbelliferone nucleus (3).

Lung cancer may be divided into two groups based on the size and appearance of the malignant cells: Non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (10). In France, NSCLC accounts for >80% of all lung tumors (10) and is comprised of three major subtypes, including adenocarcinoma, squamous and large-cell carcinoma. Large-cell lung carcinoma (13% of all lung cancer cases) has the poorest prognosis of the three subtypes, with a 4-year mortality rate of 93% compared with 88% for adenocarcinoma and 85% for squamous cell lung carcinoma (10). The cytotoxic/anti-proliferative effects of Umb have previously been demonstrated in lung cancer cell lines (2). Using MTT assay in combination with flow cytometry, it was demonstrated that Umb has more potent cytotoxicity against QU-DB cells, a large-cell lung cancer cell, compared withA549 adenocarcinoma cells (2).

Despite the dominant cytotoxic activities of Umb against certain cancer cell lines (2,3,8), its proliferative activities have been observed in normal lymphocytes (2) and at lower doses in a colorectal cancer cell line in culture media (11), indicating that this compound may have different levels of cytotoxicity against different cancer cell lines *in vitro*.

Although a number of studies are available regarding Umb anti-tumor activities, data regarding the molecular targets of Umb are limited. Proteomic studies that have performed high-throughput analyses of cell proteomes have identified biomarkers and therapeutic targets in various forms of cancer. As the most widely used method in proteomics, two-dimensional gel electrophoresis (2-DE) coupled with mass spectrophotometry (MS), has demonstrated great promise for the identification of molecular targets of cancer drugs (12).

The present study aimed to identify the molecular targets of Umb in two human lung cancer cell lines. To fulfill this, the protein targets of Umb were investigatedusing2-DE coupled with liquid chromatography (LC) -MS/MS in QU-DB large cell carcinoma and A549 adenocarcinoma cell lines, the two cancer cell lines whose *in vitro* proliferation rate were previously investigated following treatment with Umb (2).

Materials and methods

Preparation of Umb and cell culture. The synthesis of Umb was performed as previously described (13), and its purity was verified by nuclear magnetic resonance. For its addition to culture, Umb was dissolved in dimethyl sulfoxide (DMSO) and diluted with RPMI-1640 supplemented with 10% fetal bovine serum (Biosera, Ringmer, UK) prior to use. Human QU-DB and A549 lung cancer cell lines (Pasteur Institute of Iran, Tehran, Iran) were cultured at 37˚C in an atmosphere of 5% CO_2 and treated with either Umb (IC_{30} value, ~31 μM) or DMSO (0.15%) for 48 h, as test or control groups, respectively, as previously described (2).

2-DE, gel staining and scanning. Sample preparations and 2-DE were performed according to methods described previously (14). Briefly, cells were solubilized in a lysis buffer. Following 2 h incubation at room temperature, the supernatant was collected, aliquoted and stored at -70˚C. Protein concentration was determined using the Bradford method (15). Immobilized non-linear pH 3-10 gradient strips (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) were used for the first dimension, in which proteins are separated based on their isoelectric points (14). Isoelectric focusing (IEF) was performed on a PROTEAN® IEF Cell system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The second dimension, in which proteins are separated based on their molecular mass, as performed on a PROTEANII xi 2-D Cell system (Bio-Rad Laboratories, Inc.).

Analytical silver staining (mass incompatible) and preparative silver staining (mass compatible) were performed as previously described (16). The gels were scanned with a GS-800 Calibrated Densitometer (Bio-Rad Laboratories, Inc.), and the volume of each spot was subsequently analyzed by Prodigy SameSpots version 1.0 software (Nonlinear Dynamics, Newcastle, UK) to quantify the protein expression levels.

LC-MS/MS. The obtained spots were destained in 30 mM potassium ferricyanide: 100 mM sodium thiosulfate (1:1) for 10 min. In-gel digestion was performed as previously described (16). LC-MS/MS was performed using an Agilent 1100 Series LC/MSD Trap XCT (Agilent Technologies, Inc., Santa Clara, CA, USA). Each sample (25 μl) was separated on a Zorbax® 300SB-C18 column (75 μm, 150 mm; Agilent Technologies, Inc.) Protein identification was performed using the Agilent Spectrum MILL MS proteomics workbench (Agilent Technologies, Inc.).

Statistical analysis. The statistical differences in protein spot expression between treated and untreated groups were calculated using (Prodigy Software version 1.0). The spot groups which exhibited a >1.5 fold increase in expression after treatment were considered as differentially expressed proteins. $P<0.05$ was considered to indicate a statistically significant difference. All experiments were performed in triplicate.

Results

Differentially-expressed protein targets. 2-DE and LC-MS/MS were used to identify protein targets in Umb-treated QU-DB and A549 cells in comparison with corresponding DMSO-treated control cells. Three gels were run for each group. In total, 30 differentially-expressed protein targets were identified in the two Umb-treated human lung cancer cell lines compared with their DMSO controls.

Differentially-expressed proteins in the Umb-treated QU-DB cells. In gels derived from the QU-DB cells, collectively, 45 reproducible, distinct spots were observed to be differentially-expressed. Due to limitation in MS availability, 20 intense spots were picked up from gels and subjected to MS. Of these 20 spots, 8 spots were successfully identified by MS (Table I). A number of these 8 spots were mixture of several proteins, resulting in the identification of 14 different proteins. A total of 12 proteins were downregulated and two upregulated. The locations of these identified spots on the QU-DB gels are presented in Fig. 1.

The data demonstrated that Umb downregulated the production of heat shock protein 90 kDa (HSP90), HSP27, endoplasmin (two spots), vimentin (two spots), heterogeneous nuclear ribonucleoproteins (hnRNP) C1/C2, transitional endoplasmic reticulum ATPase (p97/VCP), NADH dehydrogenase [ubiquinone] iron-sulfur protein-3 (NDUFS3), importin-α2, importin-β1, tubulin α-1B, FK506-binding protein (FKBP4) and splicing factor 3A subunit-3 (SF3a3) in the QU-DB cells. By contrast, Nipsnap1 and glycine-tRNA ligase (GRS) were upregulated in the Umb-treated QU-DB cells.

Differentially-expressed proteins in the Umb-treated A549 cells. In the A549 cells collectively, 70 reproducible, distinct spots were differentially-expressed. Of these, 42 intense spots were picked up from gels, of which 16 were identified by MS (Table II). The locations of these spots on the A549 gels are presented in Fig. 2.

Of the 16 identified spots, 9 were downregulated. These included cyclophilin B, adenine phosphoribosyl transferase (APRT), dimethylarginine dimethylaminohydrolase-2

Table I. Descriptions of the identified differentially-expressed protein spots in umbelliprenin-treated QU-DB compared with dimethyl sulfoxide-treated control cells.

Spot no.	Protein name	Accession no.[a]	pI/MW, kDa	Score[b]	Function	Location	Expression
57	HSP90-β	P08238	4.97/83.26	92.18	Chaperone function	Mitochondria, cytoplasm	↓
57	Transitional endoplasmic reticulum ATPase (p97/VCP)	P55072	5.14/89.32	84.93	ATPases, cell cycle	Cytoplasm	↓
60	Vimentin	P08670	5.06/53.65	73.97	Intermediate filaments	Cytoskeletal component	↓
60	Splicing factor 3A subunit-3 (SF3a3)	Q12874	5.27/58.84	71.64	Splicing factor	Nucleus speckle	↓
60	Importin α-2	P52292	5.25/57.86	60.73	Nucleo-cytoplasmatic transport	Cytoplasm, nucleus	↓
61	HSP27	P04792	5.98/22.78	67.2	Chaperone function	Cytoplasm, nucleus	↓
61	NADH dehydrogenase [ubiquinone] iron-sulfur protein-3 (NDUFS3)	O75489	6.98/30.24	54.7	Transfer of electrons	Mitochondrion inner membrane	↓
75	Importin β-1	Q14974	4.68/97.17	46.4	Nucleo-cytoplasmatic transport	Cytoplasm, nucleus	↓
75	Endoplasmin (GRP94)	P14625	4.76/92.46	36.75	Molecular chaperone	Endoplasmic reticulum lumen	↓
77	Heterogeneous nuclear ribonucleoproteins C1/C2 (hnRNP C1/C2)	P07910	4.95/33.67	103.56	Component of ribonucleosomes	Nucleus	↓
77	Endoplasmin (GRP94)	P14625	4.76/92.46	42.22	Molecular chaperone	Endoplasmic reticulum lumen	↓
82	FK506-binding protein (FKBP4)	Q02790	5.35/51.8	166.07	Co-chaperoneactivities	Cytoplasm, nucleus	↓
82	Vimentin	P08670	5.06/53.65	86.48	Intermediate filaments	Cytoskeletal component	↓
82	Tubulin α-1B chain	P68363	4.94/50.15	70.43	Microtubules, motility	Cytoplasm, cytoskeleton	↓
50	NipSnap1	Q9BPW8	9.35/33.3	23.16	Ca^{2+} flux	Mitochondria	↑
73	Glycine-tRNA ligase (GRS)	P41250	6.61/83.16	27.91	Translation apparatus	Cytoplasm, mitochondria, serum	↑

[a]Swiss-Prot accession number; [b]Scores >20 were significant (P<0.05). The locations of spots are presented in Figure 1. ↓, decreased expression; ↑, increased expression; HSP, heat shock protein; GRP, glucose-regulated protein.

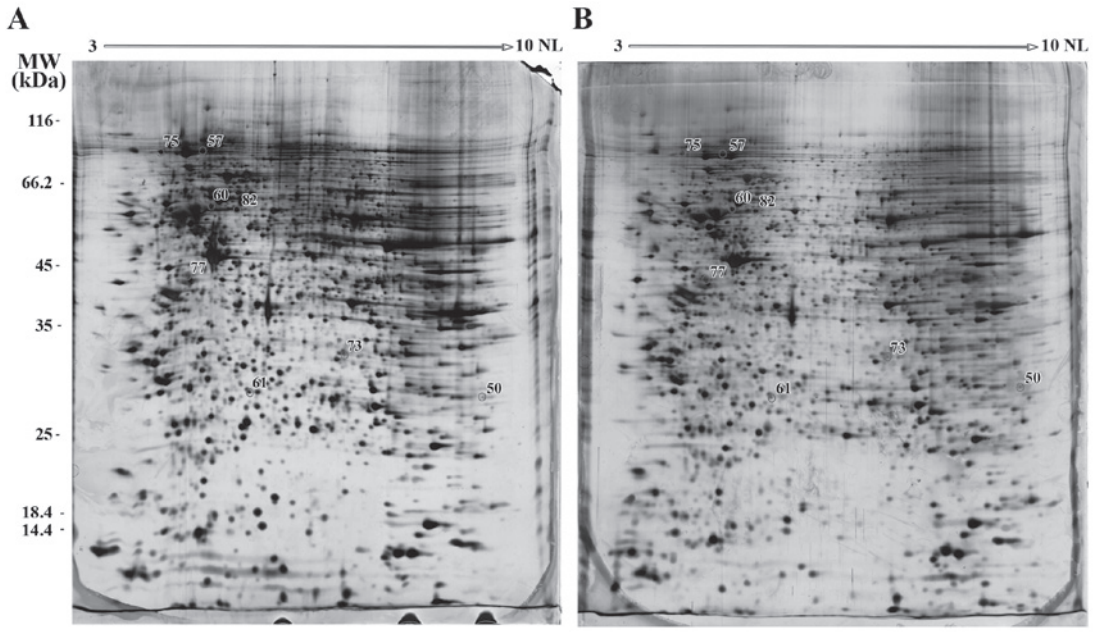

Figure 1. Proteomes of (A) dimethyl sulfoxide-treated and (B) Umb-treated QU-DB large-cell lung cancer cells were separated using two-dimensional electrophoresis and visualized by silver staining. Spot numbers are the same as those in Table I. MW, molecular; NL, non-linear.

(DDAH-2), dual specificity protein phosphatase-3 (VHR), annexin A4, prohibitin, proteasome α-1, MST and keratin-1. Data demonstrated the upregulation of 7/16 differentially-expressed proteins, which consisted of glucose-regulated protein (GRP) 78 kDa, aortic and skeletal muscle α-actins, activator of HSP90, ATPase homolog-1 (AHA1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), stathmin and calreticulin.

Discussion

To the best of our knowledge, the present study identified molecular targets of Umb in cancer for the first time using a high-throughput approach. The proteomes of the QU-DB (large-cell lung carcinoma) and A549 (adenocarcinoma) cell lines were mapped in the presence of Umb using 2-DE. The effect of Umb on these cell lines in culture media using MTT assay and flow cytometry has been previously investigated *in vitro*, with higher cytotoxicity of Umb observed against QU-DB cells (2). Furthermore, in an *in vivo* mouse model of lung cancer, a significant anti-tumor activity of Umb was reported, in addition to deviation of the immune system in favor of Th1 responses (7).

The present study demonstrated that Umb downregulated the expression of HSP90, HSP27, GRP94 (two spots), vimentin (two spots), hnRNP C1/C2, p97/VCP, NDUFS3, importin-α2, importin-β1, tubulin α-1B, FKBP4 and SF3a3 in the QU-DB cells, and cyclophilin B, APRT, DDAH-2, VHR, annexin A4, prohibitin, proteasome α-1, MST and keratin-1 in the A549 cells. The pattern of downregulated proteins suggested that Umb is an important anti-tumor compound, particularly in QU-DB cells. The functions of the proteins suggested that Umb is able to suppress tumorigenesis through different pathways, including induction of apoptosis and inhibition of cell growth, migration and angiogenesis.

In total, 12 downregulated protein spots were identified in the QU-DB cells by MS performed in the current study. The overexpression of chaperones, such as HSP90, HSP27 and endoplasmin, has been identified in various forms of cancer, including lung (17,18), and has been reported to correlate with drug resistance (18). These chaperones induce tumor cell survival as they inhibit caspase activation (18), and thus we hypothesize that Umb may induce tumor apoptosis by removing suppression from caspases. Caspases are a family of endoproteases that are critical in apoptosis (programmed cell death) with a wide range of substrates, including vimentin (19). Caspase cleavage of vimentin dislocates the cytoskeletal component of intermediate filaments and causes nuclear fragmentation in apoptotic cells (19). Vimentin was downregulated in the QU-DB cells treated with Umb in the present study. This intermediate filament is a marker of epithelial-mesenchymal transition (EMT). Vimentin overexpression has been detected in several forms of cancer and been reported as a molecular target for cancer therapy (20). hnRNPs have been linked to signal transduction, cell cycle progression and regulation of endocytosis (12). NDUFS3 is a mitochondrial Fe-S protein in electron transport chain complex I, a complex that is responsible for electron transfer to ubiquinone, and its overexpression promotes apoptotic resistance to stresses, cell proliferation, cell migration and EMT (21). VCP/p97 is an ATPase that regulates endoplasmic reticulum-associated degradation, and its inhibition induces cancer cell death (22). Importin subunits (α and β1) contribute to nucleocytoplasmic transport. Importin α, a new potential biomarker in cancer, has been demonstrated to be a predictor of poor prognosis in different forms of cancer (23). It has been reported that coumarins are able to exert anti-proliferative effects through inhibition of tubulin polymerization and induction of cell cycle arrest at the G2/M transition of the mitotic cell cycle (24). Umb may have a similar effect on QU-DB cells via tubulin reduction. FKBP4 has been

Table II. Descriptions of the 16 differentially expressed protein spots identified in umbelliprenin-treated A549 and dimethyl sulfoxide-treated control cells.

Spot no.	Protein name	Accession no.[a]	pI/MW, kDa	Score[b]	Function	Location	Expression
40	Dimethylarginine dimethylaminohydrolase-2 (DDAH-2)	O95865	5.66/29.64	39.6	Inhibitor of NOS	Cytoplasm, mitochondrion	↓
49	*St20*-like protein kinase (MST)	P25325	6.13/33.17	28.27	Catalytic enzyme, antidote	Cytoplasm	↓
51	Keratin-1	P04264	8.15/66.03	42.83	Intermediate filaments	Cell membrane	↓
58	Annexin A4	P09525	5.84/35.88	25.61	Endo- and exocytosis	Membrane-binding protein	↓
59	Proteasome α-1	P25786	6.15/29.55	17.69	Proteinase	Cytoplasm, nucleus	↓
60	Dual specificity protein phosphatase-3 (VHR)	P51452	7.66/20.47	23.51	Tyrosine phosphatase	Cytoplasm, nucleus	↓
70	Adenine phosphoribosyltransferase (APRT)	P07741	5.78/19.6	38.63	Metabolism of AMP	Cytoplasm	↓
92	Cyclophilin B	P23284	9.42/23.74	124.62	Immunophilin protein, protein folding	ER lumen, melanosome	↓
101	Prohibitin	P35232	5.57/29.8	26.6	Apoptosis-associatedprotein	Mitochondrion inner membrane	↓
16	Stathmin	P16949	5.76/17.3	27.57	Cell motility	Cytoplasm, cytoskeleton	↑
36	Actin, aortic smooth muscle	P62736	5.24/42	24.24	Cell motility	Cytoplasm, cytoskeleton	↑
38	GAPDH	P04406	8.57/36.05	13.42	Glycolysis Nuclear functions	Cytoplasm, nucleus	↑
39	Actin, α-skeletal muscle	P68133	5.23/42.05	158.76	Cell motility	Cytoplasm, cytoskeleton	↑
57	Calreticulin	P27797	4.29/48.14	62.8	Calcium-binding chaperone	Endoplasmic reticulum	↑
69	Glucose-regulated protein78 kDa	P11021	5.07/72.33	60.92	ER chaperone	Endoplasmic reticulum	↑
77	Activator of heat shock 90 kDa protein ATPase homolog-1 (AHA1)	O95433	5.41/38.27	15.5	Co-chaperone, Activities HSP90	Cytoplasm, cytosol. endoplasmic reticulum	↑

[a]Swiss-Prot accession number. [b]Scores >20 were significant ($P<0.05$). The spot numbers are the same as in Figure 2. ↓, decreased expression; ↑, increased expression; NOS, nitrous oxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

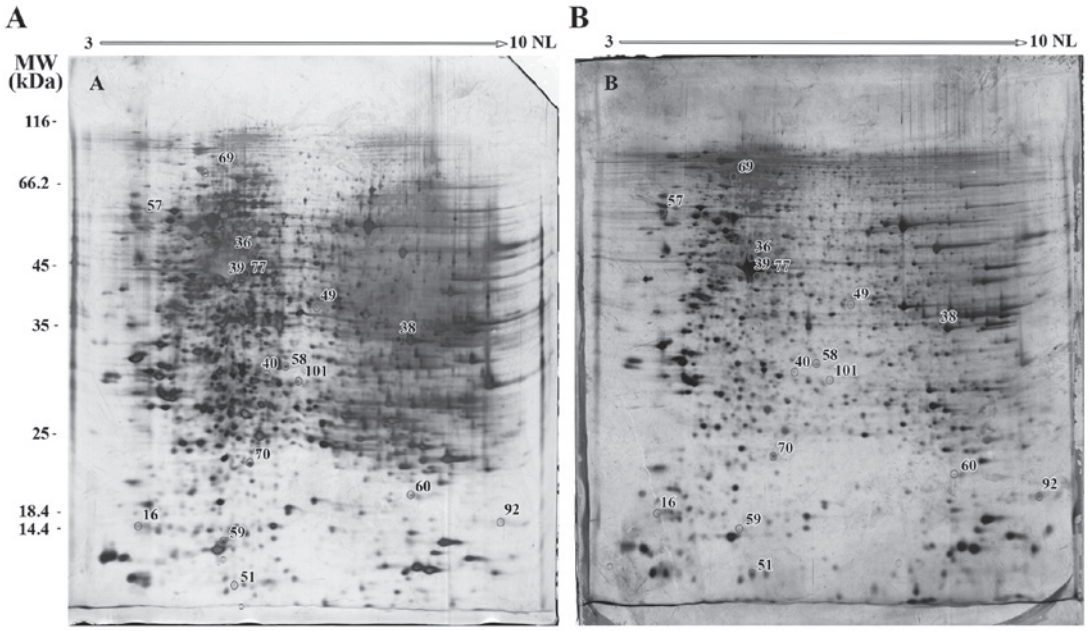

Figure 2. Proteomes of (A) dimethyl sulfoxide-treated and (B) Umb-treated A549 adenocarcinoma cells were separated using two-dimensional electrophoresis and visualized by silver staining. Spot numbers are the same as those in Table II. MW, molecular weight; NL, non-linear.

associated with a poorer prognosis in several types of cancer, including ovary, prostate and breast cancer (25), however, its role in lung cancer requires further clarification. Finally, the function of SF3a3 in cancer has not yet been clarified.

In the present study, 7 of the downregulated proteins in the A549 cells were identified by MS, and with the exception of MST, their downregulation favored the anti-tumor activity of Umb. Cyclophilin B is a ubiquitously expressed protein in normal cells and tissues, but protects cancer cells against apoptosis induced by cisplatin, hypoxia and oxidative stresses (26). The protein increases tumor growth, angiogenesis and resistance to therapy (26). Adenine is converted to AMP by the ubiquitous enzyme APRT (27). Specific inhibitors of APRT induce cytotoxicity, and have been consideredfor the treatment of cancer, arthritis, inflammation or microbial infections (27). DDAH-2 functionsas an inhibitor of nitric oxide (NO) synthase. NO overexpression has been reported in various tumors, including lung cancer, and its inhibition is regarded as an anticancer therapy (28). Cancer cells are able to evade cell cycle arrest via VHR overexpression (29). Annexins, prohibitin and different subunits of the proteasome complex have been associated with cancer invasiveness (12,13). MST is a serine/threonine-specific protein kinase and serves a conserved role as a regulator of organ size and as a potential tumor suppressor within the Hippo pathway (30). This proteins downregulation may also accelerate cancer. The role of Keratin-1 in cancer requires further clarification.

In the current study, proteins upregulated in the QU-DB cells includedNipsnap1 and GRS, while upregulated proteins in the Umb-treated A549 cells included GRP78, α-smooth muscle actin, skeletal muscle α-actin, AHA1, GAPDH, stathmin and calreticulin. In contrast to the QU-DB cells, upregulated proteins in the A549 cells may deteriorate cancer conditions and induce tumor cell survival, while only calreticulin overexpression appears to induce appropriate anti-tumor and anti-proliferative responses. Upregulated proteins in the QU-DB cells were consistent with Umb anti-tumor activities. A previous study identified Nipsnap1 as a tumor suppressor in prostate cancer (31). Immune stimulatory activity of GRS indicates its therapeutic potential against tumorigenesis. GRS is a component of the translation machinery, and is secreted from macrophages in response to Fas ligand, which is released from tumor cells (32). GRS binds to various extracellular signal-regulated kinase (ERK)-activated tumor cells and releases phosphatase 2A, which in turn suppresses ERK signaling through dephosphorylation of ERK and induces apoptosis (32). A previous study demonstrated that following *in vivo* administration of GRS, growth of tumors with a high level of ERK activation was strongly suppressed (32).

In the present study, 6 out of 7 upregulated proteins in the A549 cells were in favor of tumor growth. GRP78 is an endoplasmic reticulum (ER) chaperone, which participates in the folding of proteins in the ER (33). GRP78 stimulates tumor survival, metastasis and resistance to various therapies (33). GRP78 is considered as a novel biomarker in cancer treatment and therapeutic regimen (33). Actins are potential mediator of tumor development. In the current study, it was observed that two different types of actins, α-smooth muscle actin and skeletal muscle α-actin, were upregulated in the A549 cells. α-smooth muscle actin overexpression is a major characteristic of cancer-associated fibroblasts and serves a vital role in lung cancer initiation and progression (34). Thus, this suggests that actin polymerization and cytoskeletal rearrangement were induced in the A549 cells in response to Umb. AHA1 (18), a co-chaperone that binds to HSP90, GAPDH (14) and stathemin (35) are well-known for their roles in tumor progression. Calreticulin is a Ca^{2+}-binding chaperone and its cell surface expression is able to induce innate immune responses via 'eat me' signals, which lead to capture of dying tumor cells by dendritic cells and macrophages (36). It has been reported that

calreticulin functions as a damage-associated molecular pattern (DAMP) following tumor therapy (36). In tumor cells, calreticulin in complex with cluster of differentiation 91 functions as a macrophage surface receptor for collectin and complement factor C1q (36). This interaction induces phagocyte binding of apoptotic cells and innate immune response development (36). Calreticulin also takes part in major histocompatibility class I assembly and presentation of tumor cell antigens (36). Calreticulin expression on lung cancer cell membranes is associated with tumor pathological classification and grade, and is known to be a novel prognostic factor and potential therapeutic biomarker in lung cancer (37). Despite other overexpressed proteins identified in the Umb-treated A549 cells, it appears that only calreticulin is able to induce A549 cell repression.

By using a proteomic approach, the present study observed that targets of Umb were different in two different lung cancer cell lines. In the QU-DB cells, the targets supported its anti-tumor activities, while in the A549 cells, the targets had contradictory effects in tumorigenesis. The proteomic data are consistent with previous studies that demonstrated that a similar dosage of Umb had an apoptotic effect on QU-DB cells, but not on A549 cells, and only after increasing the Umb dose was cytotoxicity observed in the A549 cells (2). Notably, certain upregulated proteins observed in each cell line in the current study (GRS in the QU-DB cells and calreticulin in the A549 cells) are known to interact with immune cells *in vivo* and boost anti-tumor immune response (32,36,37). This may indicate that Umb has stronger *in vivo* anti-tumor activities compared to *in vitro* results.

In conclusion, to the best of our knowledge, the results of the present study provide the first evidence of molecular targets of Umb in cancer obtained by high-throughput profiling. At the molecular level, Umb affects the two cancer cell lines differentially, reflecting different levels of cytotoxicity against them that is consistent with previous studies. In each cell line, immune-associated molecules were upregulated, which suggests that Umb has stronger *in vivo* anti-tumor activity. In addition, these results highlighted the possible administration of Umb in lung cancer in an individualized manner. Whether Umb functions in the same manner within other types of lung cancer, or even on the same type, requires further clarification in future studies.

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