Anticancer effects of β-elemene in gastric cancer cells and its potential underlying proteins: A proteomic study

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Abstract. Gastric cancer is a common malignancy with a poor prognosis. β-elemene is a broad-spectrum anticancer drug extracted from the traditional Chinese medicinal herb *Curcuma wenyujin*. In the present study, we investigated the anticancer effects of β-elemene in gastric cancer cells and the potential proteins involved. Human SGC7901 and MKN45 gastric cancer cells were treated with different concentrations of β-elemene. Cell viability, clonogenic survival and apoptotic cell death were assessed. β-elemene inhibited viability and decreased clonogenic survival of gastric cancer cells in a dose-dependent manner. Apoptosis induction contributed to the anticancer effects. We then employed a proteomic method, isobaric tags for relative and absolute quantitation (iTRAQ), to detect the proteins altered by β-elemene. In total, 147 upregulated proteins and 86 downregulated proteins were identified in response to β-elemene treatment in SGC7901 gastric cancer cells. Among them, expression of p21-activated protein kinase-interacting protein 1 (PAK1IP1), Bcl-2-associated transcription factor 1 (BTF) and topoisomerase 2-α (TOPIIα) were validated by western blot analyses and the trends were consistent with iTRAQ results. Top pathways involved in β-elemene treatment in SGC7901 gastric cancer cells included ribosome signaling, peroxisome proliferator-activated receptors (PPARs) signaling pathway, regulation of actin cytoskeleton, phagosome, biosynthesis and metabolism of some amino acids. Collectively, our results suggest a promising therapeutic role of β-elemene in gastric cancer. The differentially expressed proteins provide further insight into the potential mechanisms involved in gastric cancer treatment using β-elemene.

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Introduction

Gastric cancer is the fourth most common malignancy in the world and the second leading cause of cancer-related mortality (1). At present, surgical resection remains the main therapeutic strategy for gastric cancer, supplemented with perioperative chemotherapy, chemoradiotherapy and/or immunotherapy (2-5). However, most patients are diagnosed with advanced gastric cancer which may have progressed beyond the curative potential of surgical operation (6,7). In addition, previous studies have demonstrated that a considerable proportion of patients receiving potentially curative resection experienced recurrences which lead to unfavorable prognosis (8,9). Adjuvant therapy, such as chemotherapy, provides rather limited survival advantage (10,11). These facts attest to the deficiency in the current strategies for treating gastric cancer and the demand for novel approaches to the management of gastric cancer.

Among various ingredients eligible for adjuvant therapy for gastric cancer, the significance of natural products, particularly the essence extracted from Chinese herbs are gaining increasing attention in basic and clinical research (12). β-elemene (1-methyl-1-vinyl-2,4-diisopropenyl-cyclohexane) is a novel anticancer agent extracted from the Chinese medicinal herb *Curcuma wenyujin* (13). In recent studies, β-elemene was shown to have diverse anticancer potential, such as inhibiting proliferation and inducing apoptosis of cancer cells, and interacting with multiple oncogenic or tumor suppressing signaling pathways in a broad spectrum of cancers (14-16). Other studies found that β-elemene could enhance tumor chemosensitivity or overcome drug resistance (17,18). In addition, β-elemene has been approved by the China Food and Drug Administration as a therapeutic drug in clinical practice where its efficacy has been exhibited when combined with first-line chemotherapy for malignant tumors (19,20). However, the mechanisms by which β-elemene is involved in tumor suppressing activities remain largely unknown.

In the present study, we examined the anticancer potential of β-elemene in the proliferation, clonogenic survival and apoptosis in SGC7901 and MKN45 gastric cancer cells. Then, in order to investigate the molecules through which β-elemene exhibited its anticancer effects and to obtain a better understanding of its therapeutic role in gastric cancer, we employed

isobaric tags for relative and absolute quantitation (iTRAQ), a high-throughput proteomic approach, to profile proteins that were differentially expressed following β-elemene treatment in gastric cancer cells.

Materials and methods

Reagents. β-elemene was obtained from Jingang Pharmaceutical Co. (Dalian, China). Annexin V-FITC/PI apoptosis detection kit was purchased from 7 Sea Pharmacy Technology (Shanghai, China). iTRAQ reagents were from Applied Biosystems (New York, NY, USA). Anti-PAK1IP1 antibody was purchased from Abcam (#ab67348; UK). Anti-TOPIIα antibody was obtained from Proteintech (#20233-1-AP; USA). Anti-BTF antibody was from BD Biosciences Pharmingen (#611726; San Diego, CA, USA).

Cell culture. The SGC7901 and MKN45 human gastric cancer cell lines were obtained from the Lab Animal Centre of the Fourth Military Medical University (Xi'an, China). Cells were cultured in RPMI-1640 medium (HyClone, USA), supplemented with 10% fetal bovine serum (Sijiqing, Huzhou City, China) at 37˚C with 5% CO_2 in a humidified atmosphere.

MTT assay. Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were seeded in 24-well plates at 5-10x10^4/well. After overnight incubation, cells were exposed to different concentrations of β-elemene for 24-72 h. Then, 50 μl MTT (5 mg/ml) was added to each well and the cells were incubated for another 4 h at 37˚C. After gentle removal of the supernatant, 500 μl dimethyl sulfoxide (DMSO) was added to each well to solubilize the purple formazan crystal. The optical density (OD) was measured using a microplate reader at 490 nm and then transformed into cell viability using the following formula: Cell viability = (OD of the experimental sample)/(OD of the control sample) x 100%.

Annexin V-FITC/PI apoptosis detection assay. To explore the effect of β-elemene on apoptotic cell death, Annexin V-FITC/PI apoptosis detection assay was used. The cells were seeded in 6-well plates at 3x10^5/well. After overnight incubation, cells were exposed to different concentrations of β-elemene for 24 h. Then, cells were collected and manipulated following the manufacturer's instructions, incubated with Annexin V-FITC and propidium iodide (PI), then analyzed using flow cytometry (FCM; BD Biosciences-Clontech, Palo Alto, CA, USA) within 30 min.

Clonogenic survival assay. Cells were trypsinized and counted. Then, 200 cells were seeded into each well of 6-well plates. After overnight incubation for attachment and recovery, the cells were treated with different concentrations of β-elemene. Ten to fourteen days after seeding, cells were washed with PBS twice, fixed with methyl alcohol for 15 min and stained with 1% crystal violet for 20 min. Colonies containing >50 cells was counted and the surviving fractions were calculated as follows: Plating efficiency (PE) = colony number of the control group/the number of cells seeding. Surviving fraction = colony number of the treated-group/(the number of cells seeding x PE). This assay was carried out in duplicate.

Protein preparation. After SGC7901 cells were treated with or without β-elemene at 30 μg/ml for 48 h, cell total protein was extracted. Protein concentration was determined using Pierce™ BCA Protein Assay (Thermo Scientific, Rockford, IL, USA). Total protein extracted from two separate experiments was mixed together for use in the subsequent proteomic analysis. Protein samples were reduced and alkylated, then added into 5-fold volume of ice-cold acetone and put in -20˚C condition overnight. Then, the precipitate was harvested by centrifugation at 25,000 x g at 4˚C for 20 min and dried in the air for 5 min. The precipitate was dissolved in 200 μl 0.5 M tetraethylammonium bromide (TEAB) and dealt with sonicate for 15 min. Finally, the supernatant was harvested and quantified.

iTRAQ proteomic analysis. One hundred micrograms of protein were taken out from each sample and digested with trypsin. Thereafter, the peptides of each sample were labeled with iTRAQ reagents respectively, according to the manufacturer's protocol (Applied Biosystems) (SGC7901-control-114 tag and SGC7901-β-elemene treated-115 tag). Then, the labeled samples were pooled and sent to fractionating using strong cation exchange (SCX) chromatography (Shimadzu LC-20AB HPLC Pump system and the 4.6x250 mm Ultremex SCX column). After elution, 20 fractions of peptides were obtained. Each fraction was then desalted by Strata-X C18 column (Phenomenex) and vacuum-dried. Each fraction of peptides was resuspended in buffer A (5% ACN, 0.1% FA) and centrifuged at 20,000 x g for 10 min to remove the insoluble substances. In each fraction, the final concentration of peptides was ~0.5 μg/μl. Five microlitres (~2.5 μg) of supernatant was loaded onto a Shimadzu LC-20AD nano HPLC by the autosampler for separation. Mass spectrometric analysis of the iTRAQ labelled peptides was performed using a Q Exactive (Thermo Fisher Scientific, San Jose, CA, USA) coupled online to the HPLC. Data processing of LC-MS/MS samples was searched against the International Protein Index (IPI) human protein database version 3.87 FASTA (91,464 sequences) using Mascot 2.3.02 software (Matrix Science, UK). When the fold-change of protein abundance was >1.2 and the P-value was <0.05, we defined this protein as differentially expressed. The identified proteins were categorized according to the Gene Ontology (GO) classification terms (http://www.geneontology.org/). GO enrichment analysis was performed to display the GO terms which the differentially expressed proteins enriched in all identified proteins.

Western blot analyses. Equal amounts of protein samples were subjected to SDS-PAGE. Proteins were transferred to the nitrocellulose (NC) membranes followed by 2 h blocking with 5% skimmed milk at room temperature. The NC membranes were sequentially incubated with primary antibodies at 4˚C overnight and secondary antibody for 1 h at room temperature. The reactions were visualized using electrochemiluminescence (ECL) detection kit (CWBIO, Beijing, China). The band quantification was performed using Image-Pro Plus 6.0 software (Media Cybernetics).

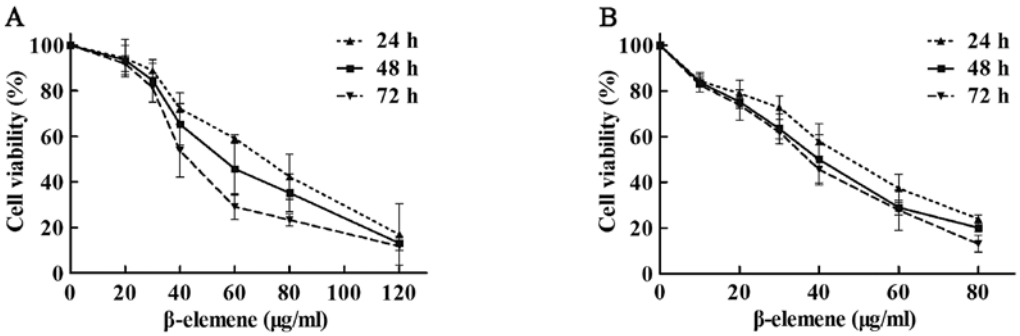

Figure 1. β-elemene inhibits the viability of gastric cancer cells. (A) SGC7901 and (B) MKN45 gastric cancer cells were treated with different concentrations of β-elemene (0, 10, 20, 30, 40, 60, 80 and 120 μg/ml) for 24, 48 or 72 h. Cell viability was determined using MTT assay. Dots and bars, means ± SD.

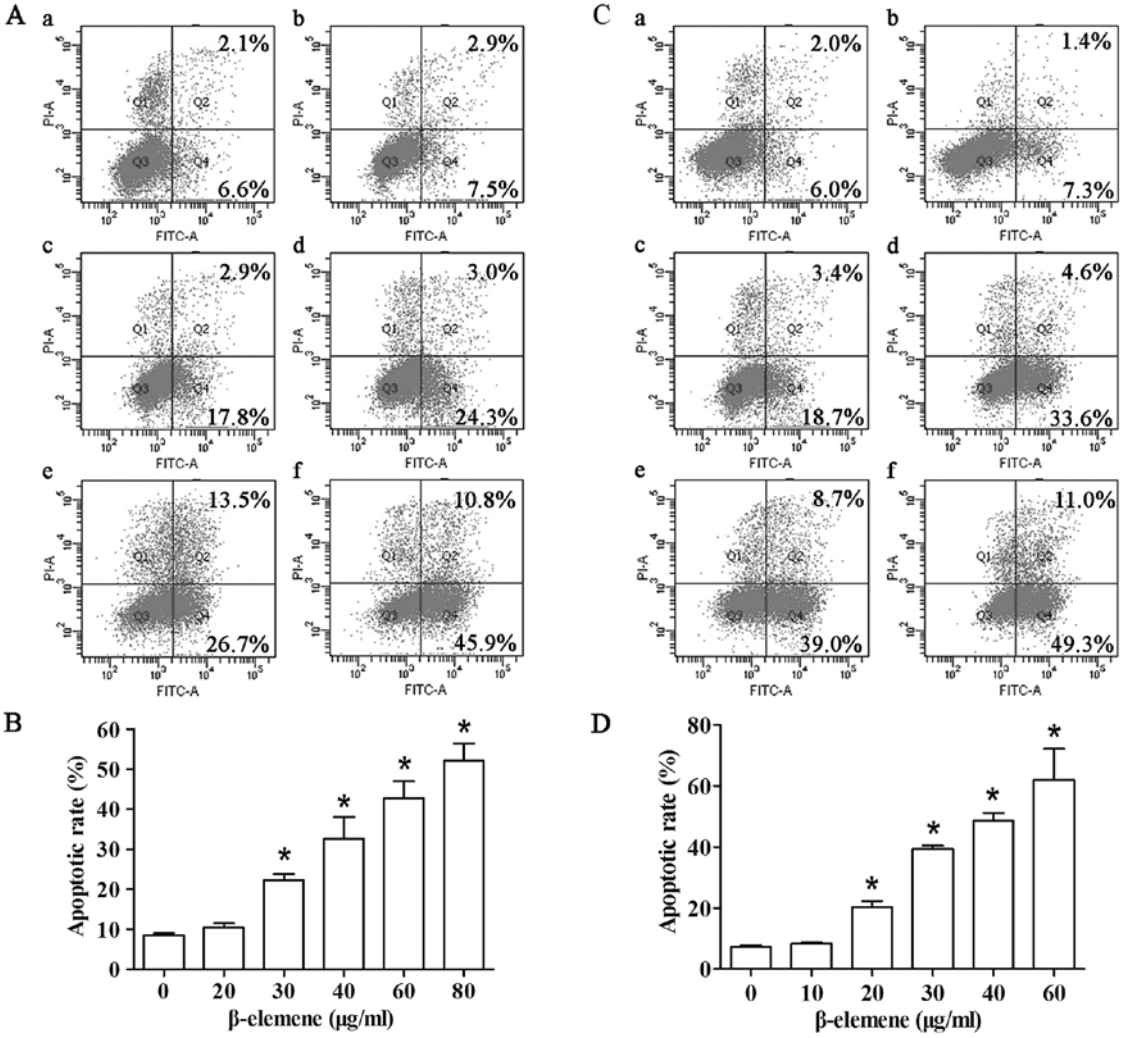

Figure 2. β-elemene induces apoptotic cell death in gastric cancer cells. Cells were treated with different concentrations of β-elemene for 24 h. Apoptotic rate was analyzed by FCM following the manufacturer's instructions. (A) Representative FCM results of SGC7901 cells. a, control; b, 20 μg/ml; c, 30 μg/ml; d, 40 μg/ml; e, 60 μg/ml; and f, 80 μg/ml. (B) Student's t-test of apoptotic rate was performed between the control group and groups treated with β-elemene in SGC7901 cells. (C) Representative FCM results of MKN45 cells. a, control; b, 10 μg/ml; c, 20 μg/ml; d, 30 μg/ml; e, 40 μg/ml; and f, 60 μg/ml. (D) Quantification of apoptosis of the control group and groups treated with β-elemene in MKN45 cells. *P<0.05. Column and bars, means ± SD. FCM, flow cytometry.

Statistical analysis. Data are presented as the means ± SD. Statistical analysis was performed using a two-tailed Student's t-test through SPSS 17.0 software (Chicago, USA). P-value <0.05 was considered to indicate a statistically significant difference.

Results

β-elemene inhibits the viability of gastric cancer cells. To investigate the antiproliferative effect of β-elemene in gastric cancer cells, SGC7901 and MKN45 cells were exposed to

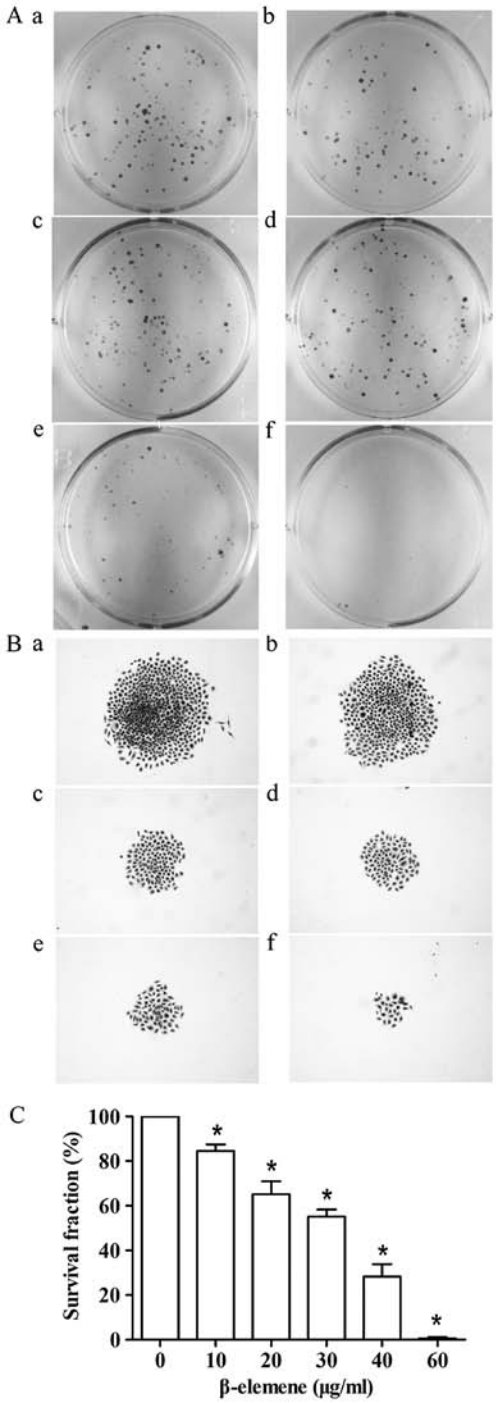

Figure 3. β-elemene inhibits clonogenic survival of SGC7901 gastric cancer cells. Two hundred single cells were seeded to each well of the 6-well plates. After attachment and recovery, cells were treated with or without β-elemene and incubated for 10-14 days to form typical colonies. (A) The entire appearance of representative wells of each experimental group. (B) Appearance of representative colony from each experimental group. (C) Clonogenic survival of the control group and groups treated with β-elemene. The results are representative of duplicate independent experiments. a, control; b, 10 μg/ml; c, 20 μg/ml; d, 30 μg/ml; e, 40 μg/ml; and f, 60 μg/ml. *P<0.05. Column and bars, means ± SD.

different concentrations of β-elemene (0, 10, 20, 30, 40, 60, 80 and 120 μg/ml) for 24, 48 or 72 h. Cell viability analysis showed that β-elemene suppressed the viability of gastric cancer cells in a dose-dependent manner (Fig. 1). The 50% inhibitory concentration (IC_{50}) values of β-elemene for SGC7901 gastric cancer cells at 24, 48 and 72 h were 67.15, 56.89 and 46.05 μg/ml, respectively. The IC_{50} values for MKN45 cells at 24, 48 and 72 h were 45.57, 37.97 and 35.29 μg/ml, respectively. These results indicate that β-elemene inhibits the viability of gastric cancer cells in a dose-dependent manner.

β-elemene induces apoptotic cell death in gastric cancer cells. FCM analysis showed that the apoptotic rate gradually increased after 24-h exposure to increased concentrations of β-elemene. The percentage of apoptotic cells in SGC7901 cells in the control group and β-elemene-treated groups (20, 30, 40, 60 and 80 μg/ml) was 8.5±0.5, 10.4±1.0, 22.2±1.6, 32.6±5.4, 42.8±4.3 and 52.1±4.3%, respectively (Fig. 2A and B). Compared with the control group, the increased rate of apoptosis reached statistical significance when the concentrations of β-elemene were >30 μg/ml in SGC7901 cells. Similar trends of apoptosis induction effects were observed in MKN45 cells (Fig. 2C and D). These data suggest that β-elemene induces apoptotic cell death in gastric cancer cells in a dose-dependent manner.

β-elemene decreases clonogenic survival of gastric cancer cells. To determine whether β-elemene inhibits colony forming efficiency, cells were exposed to different concentrations of β-elemene (0, 10, 20, 30, 40 and 60 μg/ml) and grown in a cell contact-independent manner. Consistent with the observed effects on cell viability and apoptosis-induction activity, β-elemene exhibited anti-clonogenic potential and led to a statistically significant reduction in colony formation (Fig. 3). It is worth noting that the size of the colonies tended to be smaller after treatment with β-elemene (Fig. 3B). When the concentration of β-elemene reached 60 μg/ml, the number of survived cells in each cloned cell group barely reached the standard of a colony in SGC7901 cells. These results suggest that β-elemene induces a dose-dependent inhibition of clonogenicity in gastric cancer cells.

iTRAQ identification and quantification of differentially expressed proteins by β-elemene in SGC7901 gastric cancer cells. Through iTRAQ analysis, 17,154 unique peptides corresponding to 4,267 proteins were identified in SGC7901 gastric cancer cells (data not shown). According to our definition of differentially expressed protein, a total of 233 identified proteins were regulated by β-elemene intervention in SGC7901 gastric cancer cells, including 147 upregulated proteins and 86 downregulated proteins. The altered proteins of both lists are shown in Tables I and II, respectively.

GO analysis of differentially expressed proteins altered by β-elemene in SGC7901 gastric cancer cells. Based on the GO terms analysis, categorization of these differentially expressed proteins according to cellular component, molecular function and biological process is shown in Fig. 4. GO enrichment analysis shows the top pathways involved in the differentially expressed proteins resulting from β-elemene treatment. They include phenylalanine, tyrosine and tryptophan biosynthesis, ribosome signaling, tyrosine metabolism, phenylalanine metabolism, PPAR signaling pathway, regulation of actin cytoskeleton, cysteine and methionine metabolism, ether lipid metabolism, hematopoietic cell lineage and phagosome signaling pathways.

Table I. Upregulated proteins in response to β-elemene treatment in SGC7901 gastric cancer cells.

No.	Accession	Gene symbol	Protein name	Fold-ratio (115:114)
1	IPI00549540	PAK1IP1	p21-activated protein kinase-interacting protein 1	3.967
2	IPI00152429	SPNS1	Isoform 3 of protein spinster homolog 1	2.979
3	IPI00021885	FGA	Isoform 1 of fibrinogen α chain	2.397
4	IPI00011200	PHGDH	D-3-phosphoglycerate dehydrogenase	2.319
5	IPI00006213	PCM1	Isoform 1 of pericentriolar material 1 protein	2.316
6	IPI00418426	CNNM4	Metal transporter CNNM4	2.313
7	IPI00013809	GSTZ1	Isoform 1 of maleylacetoacetate isomerase	2.292
8	IPI00012433	F8A	Factor VIII intron 22 protein	2.172
9	IPI00015856	DNPEP	Aspartyl aminopeptidase	2.144
10	IPI00002564	XRCC1	DNA repair protein XRCC1	1.981
11	IPI00909584	HARS2	Histidyl-tRNA synthetase homolog	1.922
12	IPI00218116	OASL	Isoform p30 of 59 kDa 2'-5'-oligoadenylate synthase-like protein	1.915
13	IPI00216138	TAGLN	Transgelin	1.904
14	IPI00829741	ANKLE2	Isoform 1 of ankyrin repeat and LEM domain-containing protein 2	1.872
15	IPI00022145	NUCKS1	Isoform 1 of nuclear ubiquitous casein and cyclin-dependent kinases substrate	1.771
16	IPI00290979	ABHD5	1-acylglycerol-3-phosphate O-acyltransferase ABHD5	1.761
17	IPI00554777	ASNS	Asparagine synthetase (glutamine-hydrolyzing)	1.664
18	IPI00021978	PEX11B	Peroxisomal membrane protein 11B	1.661
19	IPI00328737	ZNF598	Isoform 1 of Zinc finger protein 598	1.644
20	IPI00101782	GMPPA	Isoform 1 of mannose-1-phosphate guanyltransferase α	1.641
21	IPI00007320	TCF25	Transcription factor 25	1.627
22	IPI00031651	C7orf50	Uncharacterized protein C7orf50	1.623
23	IPI00247583	RPL21	60S ribosomal protein L21	1.622
24	IPI00329596	TMX2	Uncharacterized protein	1.613
25	IPI00740961	INTS1	DKFZP586J0619 protein	1.568
26	IPI00102752	RBM15	Isoform 1 of putative RNA-binding protein 15	1.544
27	IPI00024650	SLC16A1	Monocarboxylate transporter 1	1.539
28	IPI00026848	LRPAP1	α-2-macroglobulin receptor-associated protein	1.534
29	IPI00019976	CCDC85B	Coiled-coil domain-containing protein 85B	1.52
30	IPI00021831	PRKAR1A	cAMP-dependent protein kinase type I-α regulatory subunit	1.485
31	IPI00145260	IBA57	Putative transferase CAF17, mitochondrial	1.474
32	IPI00550021	RPL3	60S ribosomal protein L3	1.467
33	IPI00853369	PLXNB2	Plexin-B2	1.465
34	IPI00001734	PSAT1	Phosphoserine aminotransferase	1.463
35	IPI00412607	RPL35	60S ribosomal protein L35	1.449
36	IPI00027463	S100A6	Protein S100-A6	1.447
37	IPI00012772	RPL8	60S ribosomal protein L8	1.431
38	IPI00299219	CYR61	Protein CYR61	1.425
39	IPI00006362	EDF1	Isoform 2 of endothelial differentiation-related factor 1	1.423
40	IPI00007309	TIMM23	Mitochondrial import inner membrane translocase subunit Tim23	1.422
41	IPI00030362	PLP2	Isoform 1 of proteolipid protein 2	1.421
42	IPI00219840	AP2S1	Isoform 1 of AP-2 complex subunit sigma	1.42
43	IPI00980827	Unknown	Uncharacterized protein	1.419
44	IPI00296526	NAGK	N-acetyl-D-glucosamine kinase	1.417
45	IPI00465361	RPL13	60S ribosomal protein L13	1.416
46	IPI00306749	SLC4A1AP	Kanadaptin	1.409
47	IPI00168262	GLT25D1	Procollagen galactosyltransferase 1	1.404
48	IPI00410067	ZC3HAV1	Isoform 1 of Zinc finger CCCH-type antiviral protein 1	1.397
49	IPI00789805	DIAPH3	Isoform 3 of protein diaphanous homolog 3	1.395
50	IPI00293425	FXN	Isoform 1 of frataxin, mitochondrial	1.394

Table I. Continued.

No.	Accession	Gene symbol	Protein name	Fold-ratio (115:114)
51	IPI00021389	CCS	Copper chaperone for superoxide dismutase	1.376
52	IPI00027096	MRPL19	39S ribosomal protein L19, mitochondrial	1.376
53	IPI00005040	ACADM	Isoform 1 of medium-chain specific acyl-CoA dehydrogenase, mitochondrial	1.376
54	IPI00556655	LAMP1	LAMP1 protein variant (fragment)	1.375
55	IPI00101968	DBNL	Isoform 3 of drebrin-like protein	1.37
56	IPI00008418	DIABLO	Diablo homolog, mitochondrial precursor	1.36
57	IPI00396174	CCDC25	Coiled-coil domain-containing protein 25	1.357
58	IPI00010404	SF3B5	Splicing factor 3B subunit 5	1.356
59	IPI00216999	C14orf21	Pumilio domain-containing protein C14orf21	1.354
60	IPI00293276	MIF	Macrophage migration inhibitory factor	1.352
61	IPI00297241	URB1	Nucleolar pre-ribosomal-associated protein 1	1.351
62	IPI00004406	UPP1	Isoform 1 of uridine phosphorylase 1	1.347
63	IPI00023122	PDLIM7	Isoform 1 of PDZ and LIM domain protein 7	1.346
64	IPI00375731	RBM10	Isoform 1 of RNA-binding protein 10	1.345
65	IPI00170786	WBP11	WW domain-binding protein 11	1.345
66	IPI00010740	SFPQ	Isoform long of splicing factor, proline- and glutamine-rich	1.343
67	IPI00329321	LYRM7	LYR motif-containing protein 7	1.341
68	IPI00290857	KRT3	Keratin, type II cytoskeletal 3	1.34
69	IPI00456758	RPL27A	60S ribosomal protein L27a	1.339
70	IPI00063673	ISY1	Isoform 1 of pre-mRNA-splicing factor ISY1 homolog	1.339
71	IPI00217236	TBCA	Tubulin-specific chaperone A	1.339
72	IPI00301280	TMEM43	Transmembrane protein 43	1.337
73	IPI00006079	BCLAF1	Isoform 1 of Bcl-2-associated transcription factor 1	1.336
74	IPI00746351	DIS3	Isoform 1 of exosome complex exonuclease RRP44	1.33
75	IPI00180781	MLKL	Isoform 1 of mixed lineage kinase domain-like protein	1.328
76	IPI00012493	RPS20	40S ribosomal protein S20	1.321
77	IPI00008922	IFITM2	Interferon-induced transmembrane protein 2	1.32
78	IPI00647650	EIF3H	Eukaryotic translation initiation factor 3 subunit 3	1.317
79	IPI00299573	RPL7A	60S ribosomal protein L7a	1.317
80	IPI01012991	LUC7L2	Isoform 1 of putative RNA-binding protein Luc7-like 2	1.315
81	IPI00300078	PWP2	Periodic tryptophan protein 2 homolog	1.311
82	IPI00003016	STRN4	Striatin-4	1.31
83	IPI00745955	EBNA1BP2	Probable rRNA-processing protein EBP2	1.307
84	IPI00220527	SNX1	Isoform 1A of sorting nexin-1	1.305
85	IPI00017448	RPS21	40S ribosomal protein S21	1.3
86	IPI00002135	TACC3	Transforming acidic coiled-coil-containing protein 3	1.296
87	IPI00011635	BCL2L13	Isoform 2 of Bcl-2-like protein 13	1.291
88	IPI00157176	MEA1	Male-enhanced antigen 1	1.288
89	IPI00012756	IFIT5	Interferon-induced protein with tetratricopeptide repeats 5	1.287
90	IPI00022202	SLC25A3	Isoform A of phosphate carrier protein, mitochondrial	1.287
91	IPI00064767	ARHGAP17	Isoform 1 of Rho GTPase-activating protein 17	1.284
92	IPI00063827	ABHD14B	Isoform 1 of abhydrolase domain-containing protein 14B	1.282
93	IPI00243221	NRD1	Nardilysin isoform a	1.274
94	IPI00297160	CD44	Isoform 12 of CD44 antigen	1.272
95	IPI00014808	PAFAH1B3	Platelet-activating factor acetylhydrolase IB subunit γ	1.269
96	IPI00022694	PSMD4	Isoform Rpn10A of 26S proteasome non-ATPase regulatory subunit 4	1.264
97	IPI00011229	CTSD	Cathepsin D	1.261
98	IPI00003856	ATP6V1E1	V-type proton ATPase subunit E 1	1.255
99	IPI00291669	UBLCP1	Ubiquitin-like domain-containing CTD phosphatase 1	1.252

Table I. Continued.

No.	Accession	Gene symbol	Protein name	Fold-ratio (115:114)
100	IPI00029081	LIG3	Isoform α of DNA ligase 3	1.248
101	IPI00007797	FABP5	Fatty acid-binding protein, epidermal	1.248
102	IPI00028387	DDRGK1	Isoform 1 of DDRGK domain-containing protein 1	1.245
103	IPI00007694	PPME1	Isoform 1 of protein phosphatase methylesterase 1	1.243
104	IPI00031519	DNMT1	Isoform 1 of DNA (cytosine-5)-methyltransferase 1	1.241
105	IPI00219684	FABP3	Fatty acid-binding protein, heart	1.24
106	IPI00219153	RPL22	60S ribosomal protein L22	1.237
107	IPI00219029	GOT1	Aspartate aminotransferase, cytoplasmic	1.236
108	IPI00297178	DHX16	119 kDa protein	1.234
109	IPI00305267	GOLGA3	Isoform 1 of golgin subfamily A member 3	1.234
110	IPI00010697	ITGA6	Isoform α-6X1X2B of integrin α-6	1.233
111	IPI00396387	GNL1	Isoform 1 of guanine nucleotide-binding protein-like 1	1.233
112	IPI00000030	PPP2R5D	Isoform δ-1 of serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit δ isoform	1.233
113	IPI00007118	SERPINE1	Plasminogen activator inhibitor 1	1.23
114	IPI00021057	SLC12A4	Isoform 1 of solute carrier family 12 member 4	1.229
115	IPI00018206	GOT2	Aspartate aminotransferase, mitochondrial	1.229
116	IPI00019472	SLC1A5	Neutral amino acid transporter B(0)	1.229
117	IPI00306353	DUSP23	Dual specificity protein phosphatase 23	1.227
118	IPI00032825	TMED7	Transmembrane emp24 domain-containing protein 7	1.227
119	IPI01010270	WIZ	WIZ protein	1.225
120	IPI00006980	C14orf166	UPF0568 protein C14orf166	1.224
121	IPI00298625	LYN	Isoform LYN A of tyrosine-protein kinase Lyn	1.223
122	IPI00550523	ATL3	Atlastin-3	1.222
123	IPI00333215	TCEA1	Isoform 1 of transcription elongation factor A protein 1	1.222
124	IPI00032038	CPT1A	Isoform 1 of carnitine O-palmitoyltransferase 1, liver isoform	1.221
125	IPI00025512	HSPB1	Heat shock protein β-1	1.221
126	IPI00013468	BUB3	Isoform 1 of mitotic checkpoint protein BUB3	1.221
127	IPI00307165	TRIM47	Tripartite motif-containing protein 47	1.22
128	IPI00181396	VPRBP	Isoform 3 of protein VPRBP	1.217
129	IPI00216237	RPL36	60S ribosomal protein L36	1.217
130	IPI00550069	RNH1	Ribonuclease inhibitor	1.217
131	IPI00215995	ITGA3	Isoform 1 of integrin α-3	1.216
132	IPI00902512	PPP1CA	Serine/threonine-protein phosphatase	1.215
133	IPI00003923	UMPS	Isoform 1 of uridine 5'-monophosphate synthase	1.21
134	IPI00010414	PDLIM1	PDZ and LIM domain protein 1	1.21
135	IPI01026021	SHMT2	SHMT2 protein	1.21
136	IPI00021828	CSTB	Cystatin-B	1.21
137	IPI00060627	CCDC124	Coiled-coil domain-containing protein 124	1.209
138	IPI00218466	SEC61A1	Protein transport protein Sec61 subunit α isoform 1	1.207
139	IPI00026546	PAFAH1B2	Platelet-activating factor acetylhydrolase IB subunit β	1.207
140	IPI00011592	DYNC1LI2	Cytoplasmic dynein 1 light intermediate chain 2	1.206
141	IPI00216682	CNN3	Calponin-3	1.206
142	IPI00297900	DDX10	Probable ATP-dependent RNA helicase DDX10	1.206
143	IPI00292657	PTGR1	Prostaglandin reductase 1	1.204
144	IPI00925046	QARS	Glutaminyl-tRNA synthetase	1.204
145	IPI00339379	ARHGEF1	Isoform 2 of Rho guanine nucleotide exchange factor 1	1.202
146	IPI00294486	DUSP9	Dual specificity protein phosphatase 9	1.201
147	IPI00159899	ANKFY1	Isoform 1 of ankyrin repeat and FYVE domain-containing protein 1	1.201

Table II. Downregulated proteins in response to β-elemene treatment in SGC7901 gastric cancer cells.

No.	Accession	Gene symbol	Protein name	Fold-ratio (115:114)
1	IPI00654755	HBB	Hemoglobin subunit β	0.163
2	IPI00290380	ALPPL2	Alkaline phosphatase, placental-like	0.531
3	IPI00183695	S100A10	Protein S100-A10	0.598
4	IPI00419273	CUL4A	Isoform 1 of cullin-4A	0.631
5	IPI00410110	DHX40	Isoform 1 of probable ATP-dependent RNA helicase DHX40	0.637
6	IPI00011698	SAP18	Histone deacetylase complex subunit SAP18	0.654
7	IPI00022002	MRPS27	Mitochondrial 28S ribosomal protein S27	0.664
8	IPI00171438	TXNDC5	Thioredoxin domain-containing protein 5	0.675
9	IPI00023704	LPP	Lipoma-preferred partner	0.701
10	IPI00301503	TRA2B	Isoform 1 of transformer-2 protein homolog β	0.706
11	IPI00414836	OSTF1	Osteoclast-stimulating factor 1	0.706
12	IPI00019896	MYCBP2	*Homo sapiens* protein associated with Myc mRNA (fragment)	0.712
13	IPI00414101	TOP2A	Isoform 2 of DNA topoisomerase 2-α	0.716
14	IPI00412545	NDUFA5	NADH dehydrogenase (ubiquinone) 1 α subcomplex subunit 5	0.717
15	IPI00003935	HIST2H2BE	Histone H2B type 2-E	0.718
16	IPI00291467	SLC25A6	ADP/ATP translocase 3	0.722
17	IPI00374272	C5orf51	UPF0600 protein C5orf51	0.725
18	IPI00005087	TMOD3	Tropomodulin-3	0.732
19	IPI00008475	HMGCS1	Hydroxymethylglutaryl-CoA synthase, cytoplasmic	0.736
20	IPI00017342	RHOG	Rho-related GTP-binding protein RhoG	0.742
21	IPI00009901	NUTF2	Nuclear transport factor 2	0.745
22	IPI00298731	PPP1R10	Serine/threonine-protein phosphatase 1 regulatory subunit 10	0.746
23	IPI00020602	CSNK2A2	Casein kinase II subunit α	0.753
24	IPI00025347	EMG1	Ribosomal RNA small subunit methyltransferase NEP1	0.755
25	IPI00218962	C20orf43	UPF0549 protein C20orf43	0.755
26	IPI00014230	C1QBP	Complement component 1 Q subcomponent-binding protein, mitochondrial	0.755
27	IPI00219025	GLRX	Glutaredoxin-1	0.757
28	IPI00168479	APOA1BP	Apolipoprotein A-I binding protein	0.758
29	IPI00027705	PRIM2	Isoform 1 of DNA primase large subunit	0.758
30	IPI00219483	SNRNP70	Isoform 2 of U1 small nuclear ribonucleoprotein 70 kDa	0.765
31	IPI00220014	IDI1	Isoform 2 of isopentenyl-diphosphate δ-isomerase 1	0.766
32	IPI00434580	MYOM1	Isoform 1 of myomesin-1	0.771
33	IPI00023729	FN3K	Fructosamine-3-kinase	0.773
34	IPI00013446	PSCA	Prostate stem cell antigen	0.78
35	IPI00924816	MTPN	Myotrophin	0.781
36	IPI00033022	DNM2	Isoform 1 of dynamin-2	0.781
37	IPI00009032	SSB	Lupus la protein	0.781
38	IPI00073779	MRPS35	Isoform 1 of 28S ribosomal protein S35, mitochondrial	0.782
39	IPI00163644	OSBPL8	Oxysterol-binding protein	0.783
40	IPI00419626	MRPL55	Isoform 2 of 39S ribosomal protein L55, mitochondrial	0.789
41	IPI00006440	MRPS7	28S ribosomal protein S7, mitochondrial	0.79
42	IPI00027704	PRIM1	DNA primase small subunit	0.792
43	IPI00018768	TSN	Translin	0.795
44	IPI00876962	INF2	Isoform 2 of inverted formin-2	0.795
45	IPI00156374	IPO4	Isoform 1 of importin-4	0.795
46	IPI00017344	RAB5B	Ras-related protein Rab-5B	0.799
47	IPI00418290	MRPL14	39S ribosomal protein L14, mitochondrial	0.8
48	IPI00022820	GTF2B	Transcription initiation factor IIB	0.801
49	IPI00006579	COX4I1	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	0.801
50	IPI00217766	SCARB2	Lysosome membrane protein 2	0.801

Table II. Continued.

No.	Accession	Gene symbol	Protein name	Fold-ratio (115:114)
51	IPI00013396	SNRPC	U1 small nuclear ribonucleoprotein C	0.802
52	IPI00022977	CKB	Creatine kinase B-type	0.804
53	IPI00012074	HNRNPR	Isoform 1 of heterogeneous nuclear ribonucleoprotein R	0.808
54	IPI00216171	ENO2	γ-enolase	0.809
55	IPI00014958	PON2	Isoform 1 of serum paraoxonase/arylesterase 2	0.812
56	IPI00018288	POLR2C	DNA-directed RNA polymerase II subunit RPB3	0.812
57	IPI00010948	TRIM26	Tripartite motif-containing protein 26	0.812
58	IPI00007052	FIS1	Mitochondrial fission 1 protein	0.813
59	IPI00329625	TBRG4	Transforming growth factor β regulator 4	0.813
60	IPI00017510	MT-CO2	Cytochrome c oxidase subunit 2	0.814
61	IPI00645898	XPNPEP1	X-prolyl aminopeptidase (aminopeptidase P) 1, soluble	0.814
62	IPI00029054	NT5C2	Cytosolic purine 5'-nucleotidase	0.816
63	IPI00374970	SEPT10	Isoform 1 of septin-10	0.817
64	IPI00005948	MRI1	Isoform 1 of methylthioribose-1-phosphate isomerase	0.817
65	IPI00017526	S100P	Protein S100-P	0.817
66	IPI00026964	UQCRFS1	Cytochrome b-c1 complex subunit Rieske, mitochondrial	0.817
67	IPI00219673	GSTK1	Isoform 1 of glutathione S-transferase κ 1	0.818
68	IPI00296432	IWS1	Isoform 1 of protein IWS1 homolog	0.818
69	IPI00029697	EXOSC9	Isoform 2 of exosome complex component RRP45	0.82
70	IPI00062336	RPRD1A	Isoform 2 of regulation of nuclear pre-mRNA domain-containing protein 1A	0.822
71	IPI00179172	PPFIBP1	Isoform 2 of liprin-β-1	0.822
72	IPI00015972	COX6C	Cytochrome c oxidase subunit 6C	0.823
73	IPI00410657	RNMT	Isoform 2 of mRNA cap guanine-N7 methyltransferase	0.823
74	IPI00029019	UBAP2L	Isoform 2 of ubiquitin-associated protein 2-like	0.825
75	IPI00007188	SLC25A5	ADP/ATP translocase 2	0.826
76	IPI00292056	PIK3C2B	Phosphatidylinositol-4-phosphate 3-kinase C2 domain-containing subunit β	0.828
77	IPI00013475	TUBB2A	Tubulin β-2A chain	0.829
78	IPI00293590	MGLL	Monoglyceride lipase isoform 1	0.829
79	IPI00303568	PTGES2	Prostaglandin E synthase 2	0.829
80	IPI00027444	SERPINB1	Leukocyte elastase inhibitor	0.829
81	IPI00003765	CAPN7	Calpain-7	0.83
82	IPI00215920	ARF6	ADP-ribosylation factor 6	0.831
83	IPI00008449	FIP1L1	Isoform 3 of pre-mRNA 3'-end-processing factor FIP1	0.831
84	IPI00061178	RBMXL1	Heterogeneous nuclear ribonucleoprotein G-like 1	0.831
85	IPI00180292	BAIAP2	Isoform 5 of brain-specific angiogenesis inhibitor 1-associated protein 2	0.832
86	IPI00029468	ACTR1A	α-centractin	0.833

Validation of iTRAQ results by western blot analyses. Western blot analyses were performed to validate the differentially expressed proteins discovered by iTRAQ proteomic analysis. The SGC7901 human gastric cancer cells were treated the same as for the iTRAQ analysis and lysed for protein samples. Three proteins were selected for validation purposes according to our interests and the availability of antibodies. The results were consistent with those found using iTRAQ (Fig. 5) and indicated the high reliability of our iTRAQ results.

Discussion

Previous studies have shown that β-elemene, a promising anticancer drug extracted from natural plants, has efficient growth inhibition effects in a broad range of cancer cells, although with slight toxicity to normal tissue cells (14,21,22). In China, it has been used as a therapeutic candidate for certain malignant tumors for several years (20). However, little is known about the underlying molecules. In the present study, our data indicated that β-elemene efficiently suppressed the proliferation

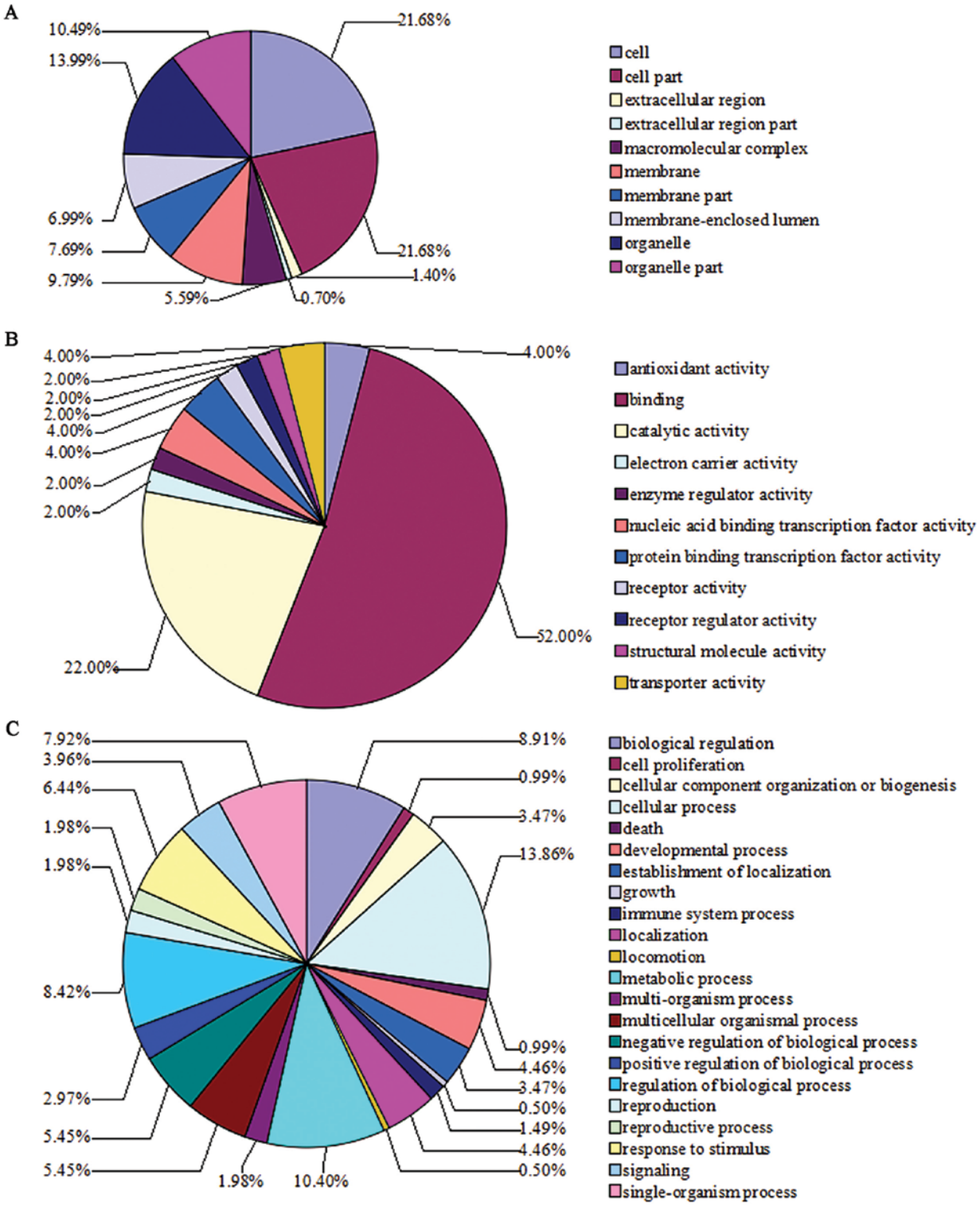

Figure 4. Categorization of the differentially expressed proteins after β-elemene treatment in SGC7901 human gastric cancer cells according to the GO terms system. (A) Protein classification according to the cellular component. (B) Protein classification according to molecular function. (C) Protein classification according to biological process. GO, Gene Ontology.

and survival of gastric cancer cells at least partly through the induction of apoptosis. The effects are consistent with results in other malignancies (15,21). Different from other studies, we employed an iTRAQ proteomic method to explore the potential proteins that may contribute to its anticancer effect. As a result, the differentially expressed proteins in response to β-elemene treatment in gastric cancer cells provided insight and supported the results found at the cytological level. Furthermore, the analysis provided some other molecules and signal pathways that may predict other pharmacologic actions of β-elemene that had not been studied in cancer therapy. In brief, our results provide evidence that β-elemene may be a

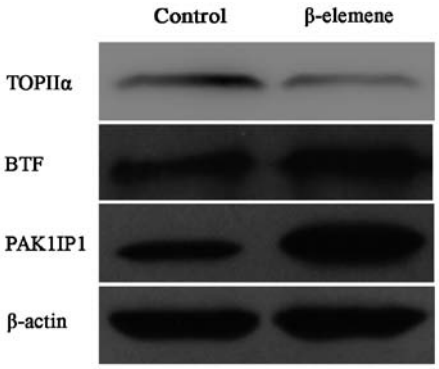

Figure 5. Validation of iTRAQ proteomic results by western blot analyses in SGC7901 gastric cancer cells. After exposure to β-elemene at 30 μg/ml for 48 h, the expression levels of PAK1IP1, BTF and TOPIIα were analyzed by western blot analyses. The trends closely match the iTRAQ proteomic results. iTRAQ, isobaric tags for relative and absolute quantitation; PAK1IP1, p21-activated protein kinase-interacting protein 1; BTF, Bcl-2-associated transcription factor 1; TOPIIα, topoisomerase 2-α.

potential drug for gastric cancer. Some of the key proteins are potential markers of gastric cancer treatment and are briefly discussed below.

Bcl-2 family proteins were found to be modulated in β-elemene-treated cancer cells in previous studies and make sense in apoptosis induction (14,21,22). The balance between pro-apoptotic and anti-apoptotic members of the Bcl-2 family proteins decides the fates of cells, and the increased proportion of pro-apoptotic proteins results in apoptotic cell death. Therefore, they play vital role in cancer therapy (23). In the present study, Bcl-2-associated transcription factor 1 (BTF) and Bcl-2-like protein 13 (also known as Bcl-rambo) were upregulated to 1.34- and 1.29-fold in response to β-elemene treatment compared with the control untreated gastric cancer cells, respectively. Both play death-promoting roles in cancer cells. BTF was first identified as a transcriptional repressor that interacted with Bcl-2 family proteins and overexpression of BTF could induce apoptosis (24). Previous studies demonstrated the roles of BTF in apoptosis promotion through the control of transcription or correlations with Bcl-2 family members (25-27). Moreover, BTF has been shown to inhibit DNA damage repair (25,27). This may partly contribute to the fact that β-elemene enhances tumor chemosensitivity or overcomes drug resistance (17,18). The other molecule, Bcl-rambo, is also a pro-apoptotic member of the Bcl-2 family (28,29). Although it was found to trigger cell death in a way distinct from the traditional Bcl-2 family members, Bcl-rambo-induced apoptotic signaling pathway eventually joined other pro-apoptotic pathways at the level of caspase-3 (28). Taken together, these results indicate that Bcl-2 family proteins play a critical role in β-elemene-induced cell death in gastric cancer cells.

p21-activated protein kinase-interacting protein 1 (PAK1IP1) was the most influenced protein among the list of upregulated proteins by β-elemene. P21-activated protein kinase 1 (PAK1) and PAK signal pathways have been shown to have multiple roles in cancer cell biological behaviors, such as cytoskeletal dynamics, survival, proliferation and transcription (30,31). PAK1 and its PAK family members are overexpressed or hyperactivated in several types of cancer and play a critical role in tumorigenesis and metastasis. Inhibition of PAK1 may efficiently block the transformation of cancer cells and act as a therapeutic strategy in cancer treatment (30,32,33). As a negative regulator of PAK1, PAK1IP1 specifically binds to the N-terminal regulatory domain of PAK1 and inhibits the activation of PAK1 by blocking the binding site of Rac and Cdc42, thus playing a negative role in cancer development and progression (30,31). However, little research has focused on PAK1IP1. In a recent study, Yu *et al* found that PAK1IP1 was upregulated when cancer cells were suffering from ribosomal stresses and overexpression of PAK1IP1 could inhibit proliferation via p53-MDM2 loop (34). In the present study, PAK1IP1 expression was ~3-fold upregulated in β-elemene-treated gastric cancer cells. Therefore, we hypothesize that β-elemene may upregulate PAK1IP1 expression and thus inhibit the activation of PAK1, which subsequently inhibits proliferation and induces apoptosis in gastric cancer cells.

One of the major protein groups regulated by β-elemene in SGC7901 gastric cancer cells was ribosomal proteins, with 12 ribosomal proteins upregulated and 4 ribosomal proteins downregulated. Over the last decade, some ribosomal proteins have been linked with emerging functions in cancer, in addition to protein synthesis. Ribosomal protein L5 (RPL5), along with RPL11 and RPL23, may form a complex with MDM2 oncoprotein and activate p53 through the inhibition of MDM2-mediated p53 degradation (35). RPS7 was also found to interact with MDM2 and overexpression of RPS7 increased cell apoptosis and suppressed cell proliferation after p53 activation (36). RPS14 and RPL11 were demonstrated to inhibit cell proliferation by negative regulation of c-Myc activity (37,38). In some other studies, the correlation between ribosomal proteins and drug resistance in cancer therapy was established. RPS13 and RPL23 could suppress drug-induced apoptosis and thus mediate multidrug resistance in gastric cancer cells (39). RPL35a was found overexpressed in many glioblastoma multiforme (GBM) brain tumors and led to chemotherapy resistance in GBM (40). Together with the present study, these results propose more roles of ribosomal proteins in cancer and therefore merit further attention in cancer therapy research.

S100A10 was a top molecule significantly downregulated by β-elemene in the present study. S100A10, also known as p11, is a unique member of the S100 protein family which serves for intracellular calcium signaling and is characterized by two EF hand motifs (41,42). The homodimer of S100A10 forms a heterotetrameric complex with two molecules of Annexin A2, a type of plasma membrane protein, to maintain stability and execute its functions (43). Expression of S100A10 has been detected in a broad spectrum of tissues and cancers including gastric cancer (44-47). Over the past years, increasing evidence has demonstrated the promoting role of S100A10 in tumor invasion and metastasis and that knockdown of S100A10 could efficiently suppress cancer progression (46-48). Notably, PAK1IP1, the most influenced protein of upregulated proteins by β-elemene, specifically targets PAK1 which is also most associated with cytoskeletal dynamics. Collectively, we deduced that β-elemene may inhibit invasion and metastasis in gastric cancer therapy, which warrants further investigation.

In conclusion, this is the first time that the iTRAQ proteomic method has been employed in the study of β-elemene in cancer cells. The present study indicated a promising anticancer role of β-elemene in gastric cancer therapy. The expression of a wide range of proteins was altered when gastric cancer cells were exposed to β-elemene. The differentially expressed proteins provided comprehensive insight into the potential underlying molecular mechanisms of the anticancer effects of β-elemene in gastric cancer cells. Furthermore, some of the proteins may act as predictors regarding further therapeutic potential of β-elemene, which merits further study in gastric cancer treatment. The present study was a preliminary exploration into the anticancer potential of β-elemene in gastric cancer cells. Based on the current results, we expect more research to be carried out on β-elemene and other traditional herbal medicine, in order to improve the management of gastric cancer.

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