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Original Paper

ITRAQ-Based Proteomics Analysis of Triptolide On Human A549 Lung Adenocarcinoma Cells

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Key Words

Triptolide • NSCLC • ITRAQ • Proteome

Abstract

Background/Aims: Triptolide (TP) is a diterpenoid triepoxide extracted from the traditional Chinese medical herb Tripterygium wilfordii that exerts prominent broad-spectrum anticancer activity to repress proliferation and induce cancer cell apoptosis through various molecular pathways. We previously observed that TP inhibits the progression of A549 cells and pancreatic cancer cells (PNCA-1) in vitro. However, the complex molecular mechanism underlying the anticancer activity of TP is not well understood. Methods: To explore the molecular mechanisms by which TP induces lung cancer cell apoptosis, we investigated changes in the protein profile of A549 cells treated with TP using a proteomics approach (iTRAQ [isobaric tags for relative and absolute quantitation] combined with NanoLC-MS/MS [nano liquid chromatographymass spectrometry]). Changes in the profiles of the expressed proteins were analyzed using the bioinformatics tools OmicsBean and the Kyoto Encyclopedia of Genes and Genomes (KEGG) and were verified using western blotting. Apoptosis and cell cycle effects were analyzed using flow cytometry. *Results:* TP induced apoptosis in A549 cells and blocked A549 cells at the G2/M phase. Using iTRAQ technology, we observed 312 differentially expressed proteins associated in networks and implicated in different KEGG pathways. Gene Ontology (GO) analysis showed the overviews of dysregulated proteins in the biological process (BP), cell component (CC), and molecular function (MF) categories. Moreover, some candidate proteins involved in PARP1/AIF and nuclear Akt signaling pathways or metastasis processes were validated by western blotting. Conclusion: TP exerted anti-tumor activity on non-small cell lung cancer (NSCLC) A549 lung adenocarcinoma cells by dysregulating tumor-related protein expression. Herein, we provide a preliminary study of TP-related cytotoxicity on A549 cells using proteomics tools. These findings may improve the current understanding of the anti-tumor effects of TP on lung cancer cells and may reveal candidate proteins as potential targets for the treatment of lung cancer.

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Introduction

Lung cancer is one of the most prevalent malignancies in humans, and its incidence and mortality are increasing worldwide [1]. Approximately 80% of lung cancers are nonsmall cell lung cancers (NSCLCs). Although diagnostic and treatment methods have recently improved markedly, no significant improvements in the prognosis of patients with NSCLC have been achieved, as the 5-year survival rates for all patients diagnosed with lung cancer remain at approximately 15% [2, 3]. Currently, the major methods for lung cancer treatment include surgery, chemotherapy and radiotherapy. Among these methods, chemotherapy can significantly relieve symptoms and improve patient quality of life. However, the efficacy and safety of this treatment remain a primary concern. Chemotherapy drugs have some serious side effects, and the toxicity of these drugs has always been an obstacle to clinical applications [4]. Therefore, the development of new therapeutic drugs for lung cancer that have high efficacy and low toxicity is clinically important.

Triptolide (TP), originally extracted from the traditional Chinese medicinal plant *Tripterygium wilfordii* [5], has been confirmed to have myriad biological properties, including immunosuppression and anti-inflammatory effects, and has been applied for the treatment of autoimmune diseases, such as nephritis and rheumatoid arthritis [6-8]. Recently, numerous studies have demonstrated that TP possesses prominent anti-tumor activities in diverse tumor cell types *in vitro*, such as breast [9], pancreatic [10], ovarian [11], lung [12], and prostate cancers [13]. TP can also prevent tumor growth *in vivo* via cell proliferation inhibition and apoptosis induction [14]. Moreover, studies have reported that TP sensitizes human cancer cells to cisplatin, 5-fluorouracil (5-FU) and TNF-alpha-induced apoptosis *in vivo* and *in vitro* [15, 16]. In a previous study, we observed that TP induces apoptosis in human lung cancer cells through PP2A-regulated ERK, p38, MAPK and Akt signaling pathways [17]. Until recently, the activity of TP in inducing tumor cell death has been well documented, but the complex molecular targets of TP anti-tumor activity have not been well characterized. Thus, a powerful tool to accurately monitor and quantitatively detect changes in protein expression in response to TP treatment is needed.

Proteomics approaches, enabling relatively comprehensive global analyses, have been widely used to examine complex biological functions [18-20]. The isobaric tags for the relative and absolute quantitation (iTRAQ) method combined with nano liquid chromatographymass spectrometry (NanoLC-MS/MS), developed for protein quantitation, represent a high-throughput quantitative technique with high sensitivity and reproducibility. Currently, iTRAQ-based proteomics has been widely used to investigate the mechanistic effects of chemicals on cancer [21-24].

In the present study, we employed a strategy combining iTRAQ with NanoLC-MS/MS to analyze alterations in the protein profile of the A549 lung adenosquamous carcinoma cell line following TP treatment. Differential protein expression data may provide a valuable resource to reveal potential molecular targets underlying the anticancer activity of TP and to improve the understanding of the anti-tumor effects of TP on lung cancer.

Materials and Methods

Cell culture and treatment

Human lung cancer A549 cells (American Type Culture Collection; ATCC CCL185) were maintained in monolayer culture at 37°C in a humidified atmosphere with 5% CO_2 in RPMI-1640 medium (Gibco-BRL, USA) supplemented with 10% fetal bovine serum (FBS) (Sijiqin Biotechnology Co. Ltd., China) and 1% penicillin/streptomycin solution (100 U/ml penicillin and 100 µg/ml streptomycin). A total of 20 mg of TP (purity≥98%, Beijing Fan-China Biotechnology Co., Ltd.) was dissolved in 0.5 ml dimethylsulfoxide (DMSO) to obtain a 100% stock solution, which was subsequently stored at -20°C and diluted with medium prior to use in experiments. The final DMSO concentration did not exceed 0.1% (v/v) throughout the study. For



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exposure experiments, A549 cells at approximately 80% confluency were transferred to medium containing 12.5, 50, and 200 ng/ml of TP and were cultured for 36 h (24-h 50% inhibitory concentration [IC50]=273.0 ng/ml) [17]. Cells treated with an equal amount of DMSO were employed as a control, and all of the treatments were performed in triplicate. Three replicate proteomics analyses were performed for each test concentration and the control group. The iTRAQ experimental results described herein are only for the 200 ng/ml TP concentration, as cells represented more acute TP cytotoxicity at this concentration.

Flow cytometric cell cycle and cell apoptosis analysis

The effects of TP on cell cycle progression were measured using flow cytometry. The fixed cells were stained with propidium iodide (PI, ComWin Biotech Co. Ltd., China) solution (50 μ g/ml PI and 100 μ g/ml RNase A in PBS) and were subsequently subjected to cell cycle analysis. Cell apoptosis was measured using Annexin V/PI double staining (ComWin Biotech Co. Ltd., China). Briefly, 100 μ l of binding buffer containing 2.5 μ l of Annexin V-fluorescein isothiocyanate (FITC) and 1 μ l of PI was added to the cell suspension, followed by incubation for 30 min in the dark. The samples were assayed using a Beckman-Coulter Flow Cytometer with excitation at 488 nm and emission at 525 nm for FITC and 575 nm for PI. The data were analyzed using FlowJo software.

Protein preparation

The harvested cells were washed five times using ice-cold PBS and disrupted using enhanced RIPA lysis buffer (Beyotime Co., China) containing protease and phosphatase inhibitors for 30 min on ice, followed by ten cycles of 5-second bursts of sonication with 30-second intervals. The cell debris was removed by centrifugation at 12, 000×g for 30 min at 4°C, and the supernatants were collected. Protein concentrations were assayed using a bicinchoninic acid (BCA) protein assay kit according to the manufacturer's instructions (Beyotime Co., China). Bovine serum albumin (BSA) was used as the standard.

iTRAQ labeling and high-pH RPLC fractionation

The iTRAQ Reagent 4-Plex kit (AB Sciex, USA) was used according to the manufacturer's instructions to label peptides. Equal amounts of protein (100 μ g per sample) obtained from TP-treated and control cells were labeled using iTRAQ labeling reagents. The TP-treated samples were labeled using 117, while the control samples were labeled using 114. Briefly, the proteins in each sample were reduced with dithiothreitol (DTT) and were subsequently alkylated with iodoacetamide. The samples were digested overnight at 37°C using trypsin (AB Sciex, USA) at a trypsin:protein ratio of 1:20 (W/W). The tryptic peptides were labeled using iTRAQ reagents. The labeled samples were combined and lyophilized. The peptide mixtures were dissolved in high-pH reverse phase (HP-RP) solvent A (20 mM ammonium formate, pH 10.0). The peptides were fractionated using the Shimadzu LC-30A system with a Durashell-C18 column (4.6 mm×250 mm, 5 μ m 100 Å, Agela, China) for high-pH RP chromatography. A total of 40 RP fractions were collected and subsequently dried and reconstituted using 30 μ l of 0.1% FA for NanoLC-MS/MS analysis.

NanoLC-MS/MS analysis

Separation was performed using the Eksigent nanoLC-UltraTM 2D System combined with the cHiPLCTM-Nanoflex system in Trap-Elute mode connected to a Triple TOF 4600 mass spectrometer (AB Sciex, USA). Briefly, 8 μ l of each fraction was loaded onto the cHiPLC trap (200 μ m × 500 μ m ChromXP C18-CL 3 μ m 300 Å) and washed for 15 min at 2 μ l/min. Subsequently, an elution gradient of 10-43% acetonitrile (0.1% formic acid) in an 85-min gradient at 300 nl/min was used on a nano cHiPLC column (75 μ m × 15 cm ChromXP C18-CL 3 μ m 300 Å). The MS analysis was performed using a nano ion spray voltage maintained at 2.3 kV and a scan range of 350 to 1500 (m/z) in the positive-ion mode. Full-scan MS spectra were acquired from 40 precursors selected for MS/MS from an m/z 100-1500 range using a dynamic exclusion setting of 30 s. The IDA CE parameter script, which selected up to 40 precursors with charge states of 2+ to 4+, automatically controlled the collision energy (CE). The mass spectrometer was calibrated using the tryptic peptides of beta-galactosidase.

Protein identification and quantitation

Peptide identification and quantification were conducted using ProteinPilot 4.2 software (AB Sciex, USA). The following search parameters were used: (1) sample type, iTRAQ 4-plex (Peptide Labeled); (2)



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cysteine alkylation, iodoacetamide; (3) digestion, trypsin; (4) instrument, Triple TOF 4600; (5) special factors, none; (6) species, Homo sapiens; (7) ID Focus, biological modifications; (8) database, UniProtKB/ Swiss-Prot FASTA; and (9) search effort, Thorough ID. In the iTRAQ quantitation, the Pro Group algorithm was automatically selected to calculate the reporter-peak areas. To estimate the false discovery rate (FDR) for peptide identification, a decoy database search strategy was adopted. For this study, a strict unused confidence score of >1.3 was used as the qualification criterion, corresponding to a peptide confidence level of 95%. If the iTRAQ ratios were >2.0 or <0.5 in the samples obtained from the TP-treated A549 cells relative to those of the control group, then the proteins were considered differentially expressed.

Bioinformatics analysis of proteomics data

The identified proteins were classified according to annotations from the UniProt knowledge base (Swissprot/TrEMBL, http://www.uniprot.org/).The multi-omics data analysis tool, OmicsBean, was used to analyze the obtained proteomics data (http://www.omicsbean.com), in which distributions in biological process (BP), cellular components (CCs) and molecular functions (MF) were assigned to each protein based on Gene Ontology (GO) categories. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (http://www.genome.ad.jpkegg/pathway.html) was performed to enrich high-level functions in the defined biological systems. Protein-protein interaction (PPI) analysis was performed using Cytoscape software, with a confidence cutoff of 400; interactions with larger confident scores are indicated with solid lines between genes/proteins, or otherwise shown as dashed lines.

Western blotting

The A549 cells were washed twice with cold PBS and lysed in 200 μ l RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate; Beyotime Co., China) containing 100 mM phenylmethanesulfonyl fluoride (PMSF; Beyotime Co., China) for 30 min on ice. The lysates were centrifuged at 14, 000×g for 10 min at 4°C, and the supernatants were collected. Twenty micrograms of protein/well was loaded onto 10% gels for separation using sodium dodecyl sulfatepolyacrylamide gels electrophoresis (SDS-PAGE). The gels were electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes (0.45 or 0.20 μ m pore size; Millipore, Billerica, MA, USA). The blotted membranes were blocked with 5% nonfat dry milk in a Tris-buffered saline solution (25 mM Tris, pH 7.5, and 150 mM NaCl) containing 0.05% Tween 20 (TBST) for 2 h at room temperature, followed by incubation with the diluted primary antibody against target protein for 4 h at room temperature. After washing for 10 min in TBST solution, the membranes were incubated with properly diluted secondary antibody conjugated with horseradish peroxidase for 2 h at room temperature. Western signals were developed using ECL chemiluminescent reagents from Thermo Scientific (Waltham, MA, USA). The β -actin levels were used as loading controls.

Statistical analysis

Western blotting and flow cytometry results are presented as the means \pm standard deviations (SD) from three independent experiments. Statistical analysis of the quantitative data for multiple group comparisons was performed using one-way ANOVA. Duncan's test (two sided) was used to determine the statistical significance levels (*P*<0.05 and *P*<0.01) between control and TP-treated groups.

Results

TP-induced apoptosis of A549 cells

To investigate the effects of TP on apoptosis in A549 cells, Annexin V/PI staining-based FACS analysis was performed to detect the externalization of phosphatidylserine on the cell membrane, a hallmark of early apoptosis. Cells undergoing early-stage apoptosis were stained with Annexin V-FITC+/PI-, and late apoptotic cells were stained with Annexin V+/PI+. Fig. 1A quantifies the increase in early apoptotic cells labeled with Annexin V+/PI-, showing increases from 3.2% in the control group to 14.8, 20.4% and 27.3% in the TP-treated groups, respectively. In addition, the overall apoptotic rates were significantly increased in the TP groups. These findings revealed that various TP treatments induced significantly higher



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percentages of apoptotic cells than the control group (Fig. 1B), indicating that TP can promote apoptosis.

Effects of TP on cell cycle arrest

The effects of TP on the cell cycle are shown in Fig. 2. Compared with the control group, the percentage of cells in the G0/G1 phase decreased from 63.84% to 13.75%, whereas the percentage of cells in the G2/M phase increased from 14.66% to 64.56%. These results indicated that TP blocks A549 cells at the G2/M phase.

Comparative proteomics of TP-treated A549 cells versus control A549 Cells

The proteins were extracted from cells treated in parallel. The samples were digested using trypsin and were labeled using 114 and 117 iTRAQ tags, and the labeled digests were utilized for MS analysis (Fig. 3A). The database we searched contains 376809 entries. Using ProteinPilot 4.5, we identified totals of 4977/5102/4762 proteins in these two cell lines in the three runs (Local FDR of <5%). Using filters with an unused protein score of >1.3 and a number of peptides of ≥ 2 , 4561/4446/4302 proteins were identified (Table 1). In total, we identified up-regulated 141 (with 117:114 iTRAQ ratios of >2.0) and 171 down-regulated proteins (with 117:114 iTRAQ ratios of <0.5) in TP-treated A549 cells compared with

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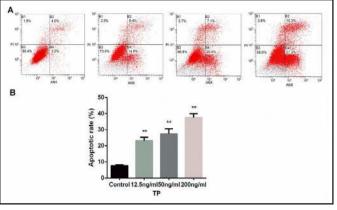


Fig. 1. Apoptotic effects of TP on human lung cancer A549 cells. (A) After 36 h of TP treatment (12.5.50 and 200 ng/ml) A549

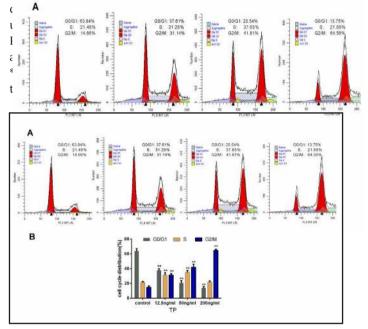


Fig. 2. Effects of TP on cell cycle distribution in human lung cancer A549 cells. (A) Representative histograms depicting cell cycle distributions in A549 cells treated with TP. After 36 h of TP exposure (0, 12.5, 50 and 200 ng/ml), A549 cells were stained with PI and analyzed using flow cytometry. (B) The percentage of the total cell population in each phase of the cell cycle is represented as a bar diagram. **P<0.01 compared with the control group. Data are presented as the means ± SD of three independent experiments.

Ermonimont	Number of distinct	Number of distinct	Number of	Unused protein
Experiment No.	proteins	peptides	spectra	score(>1.3)
INO.	Local FDR (<5%)	Local FDR (<5%)	Local FDR (<1%)	and peptide(>2)
1	4977	57223	17567	4561
2	5102	60205	17634	4446
3	4762	56342	16589	4302

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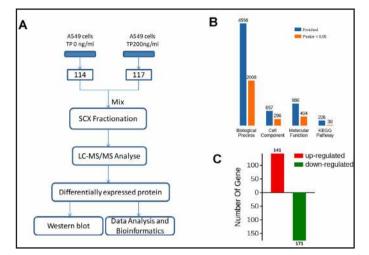
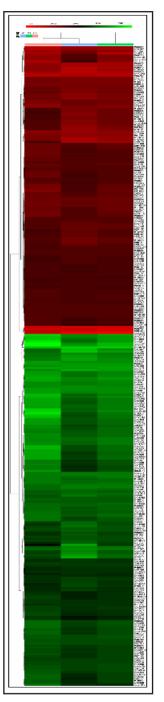


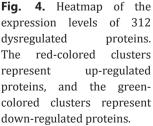
Fig. 3. Experimental quantitative proteomics analysis workflow and results. (A) Experimental design for the quantitative proteomics analysis. The proteins from A549 cells treated with different concentrations of TP were digested with trypsin and labeled using 114/117/iTRAQ tags. The labeled digests were analyzed using Nano LC-MS/MS. The differentially expressed proteins were evaluated using western blotting and analyzed through database searches. (B) The enriched counts for Biological Process, Cellular Component, Molecule Function, and KEGG Pathway. The counts for each category represent the total number of terms in the database associated with the query gene/protein list. Terms with P-values<0.05 are statistically significant. (C) In total, 312 proteins were identified, including 141 up-regulated proteins and 171 down-regulated proteins.

the control cells (Fig. 3C, Tables 2 and 3). Among those 312 dysregulated proteins, 50 proteins related to cell apoptosis were enriched (Table 4). Afterward, we performed a cluster analysis to get the heatmap which contains the data obtained for the 312 dysregulated proteins. The three horizontal clusters represent the technical replicates (Fig. 4).

Functional enrichment of the TP-regulated proteins

The obtained protein data were analyzed using bioinformatics approaches to extract information relevant to the involved pathways. Enrichments of TP-related proteins in BP, CC, and MF categories based on GO analysis are shown in Fig. 3B. In the BP analysis, the majority of identified proteins were classified into metabolic processes, particularly nitrogen compound metabolism and cellular nitrogen compound metabolism. The CC analysis showed that most of the identified proteins belonged to organelles and membrane-bounded organelles. The molecular functional classification revealed that most of these proteins were involved in binding, heterocyclic compound binding, and protein binding (Fig. 5.). GO analysis indicated that these TP-induced differentially expressed proteins exhibited a wide variety of cellular distributions and





functions, consistent with the fact that TP has broad-spectrum anti-tumor effects.



Table 2. List of up-regulated proteins in TP-treated A549 cells

Accession	Gene name	Protein name	Fold change	P-value
NO.	Gene name	riotein name	(Mean±SD)	(Mean±SD)
095831-3	AIFM1	Isoform 3 of Apoptosis-inducing factor 1	2.05±0.04	0.15±0.010
P35659	DEK	Protein DEK	2.49±0.45	0.01±0
P35232	PHB	Prohibitin	2.61±0.4	0.01±0.02
Q13464	ROCK1	Rho-associated protein kinase 1	2.75±0.61	0±0
P30101	PDIA3	Protein disulfide-isomerase A3	2.15±0.2	0±0
043399	TPD54	Tumor protein D54	2.84±0.75	0.05±0
Q9BXJ9	NAA15	N-alpha-acetyltransferase 15, NatA auxiliary subunit	2.1±0.1	0±0
F5H6E2	F5H6E2	Unconventional myosin-Ic	2.67±0.59	0.01±0
P23284	PPIB	Peptidyl-prolyl cis-trans isomerase B	2.94±0.83	0±0
Q15293	RCN1	Reticulocalbin-1	2.14±0.13	0.02±0.01
060264	SMCA5	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5	2.31±0.35	0±0
J9JID7	J9JID7	Lamin B2, isoform CRA_a	2.39±0.36	0±0
Q14257	RCN2	Reticulocalbin-2	2.13±0.06	0±0
Q32P28	P3H1	Prolyl 3-hydroxylase 1	2.05±0.05	0.02±0.02
Q08211	DHX9	ATP-dependent RNA helicase A	2.71±0.7	0±0
P09622	DLDH	Dihydrolipoyl dehydrogenase	2.56±0.34	0.02±0
E9PC15	E9PC15	Acylglycerol kinase	2.28±0.24	0±0
P09874	PARP1	Poly [ADP-ribose] polymerase 1	2.48±0.46	0±0.01
Q5JRX3	PREP	Presequence protease	2.39±0.34	0±0
Q9H3N1	TMX1	Thioredoxin-related transmembrane protein 1	2.19±0.06	0.02±0
P00167	CYB5	Cytochrome b5	2.2±0.07	0.04±0.01
P54819	KAD2	Adenylate kinase 2	2.12±0.07	0.01±0.02
075390	CISY	Citrate synthase	2.41±0.31	0±0
P21796	VDAC1	Voltage-dependent anion-selective channel protein 1	3.01±0.17	0±0
P30084	ECHM	Enoyl-CoA hydratase	2.11±0.01	0.02±0.01
Q07021	C1QBP	Complement component 1 Q subcomponent-binding protein	2.52±0.47	0.03±0.01
Q6ZMP0	THSD4	Thrombospondin type-1 domain-containing protein 4	2.14±0.07	0.04±0
000116	ADAS	Alkyldihydroxyacetonephosphate synthase, peroxisomal	2.65±0.6	0.03±0.01
P45880	VDAC2	Voltage-dependent anion-selective channel protein 2	2.93±0.69	0±0
Q9Y617	SERC	Phosphoserine aminotransferase	2.31±0.09	0±0
P61221	ABCE1	ATP-binding cassette sub-family E member 1	2.14±0.15	0±0
J3QRS3	J3QRS3	Myosin regulatory light chain 12A	2.96±1	0.01±0.02
P13667	PDIA4	Protein disulfide-isomerase A4	2.3±0.04	0±0
P25705	ATPA	ATP synthase subunit alpha	2.76±0.36	0±0
P00505	AATM	Aspartate aminotransferase	2.74±0.64	0±0
P33993	MCM7	DNA replication licensing factor MCM7	2.26±0.04	0.03±0.01
Q6DD88	ATLA3	Atlastin-3	2.49±0.27	0±0
Q9Y266	NUDC	Nuclear migration protein nudC	2.21±0.2	0±0 0.03±0.01
B7ZKQ9 P07237	B7ZKQ9 PDIA1	SCARB1 protein Protein disulfide-isomerase	2.53±0.25 2.54±0.23	0.03±0.01 0±0
P05556	ITB1	Integrin beta-1	2.34±0.23 2.46±0.11	0±0 0±0
	SYK			0±0 0.01±0.02
Q15046	BCAT2	LysinetRNA ligase Branched-chain-amino-acid aminotransferase	2.8±0.26	0.01±0.02 0.02±0.01
015382 Q14847	LASP1	LIM and SH3 domain protein 1	2.77±0.56 2.35±0.11	0.02±0.01 0±0
	Q5H909	Melanoma-associated antigen D2	2.53±0.11 2.51±0.13	0.02±0.01
Q5H909 P40939	ECHA	Trifunctional enzyme subunit alpha	2.97±0.13	0.02±0.01 0±0
Q16543	CDC37	Hsp90 co-chaperone Cdc37	2.52±0.08	0±0 0±0
Q16543 Q16658	FSCN1	Fascin	2.32±0.08 2.25±0.34	0±0 0±0
H0YDL9	H0YDL9			0.04±0
E9PDF6	E9PDF6	CD81 antigen (Fragment) Unconventional myosin-Ib	2.44±0.06 2.88±0.52	0.04±0 0±0
E9PDF6 P05023	AT1A1	Sodium/potassium-transporting ATPase subunit alpha-1	2.88±0.52 3.17±0.94	0±0 0±0
P03023 P02786	TFR1	Transferrin receptor protein 1	2.72±0.3	0±0 0±0
P02786 P48506	GSH1	Glutamatecysteine ligase catalytic subunit	2.72±0.3 2.49±0.03	0.01±0.01
P40500 P22570-3	ADRO	Isoform 3 of NADPH: adrenodoxin oxidoreductase	2.49±0.03 2.42±0.16	0.01±0.01 0±0
Q01650	LAT1	Large neutral amino acids transporter small subunit 1	2.42±0.10 2.68±0.17	0.01±0.02
000469	PLOD2	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2	2.86±0.17	0.01±0.02 0±0
P16152	CBR1	Carbonyl reductase [NADPH] 1	2.86±0.39 2.31±0.39	0±0 0±0
095202	LETM1	LETM1 and EF-hand domain-containing protein 1	2.31±0.39 2.61±0.03	0±0 0±0
P23368	MAOM	NAD-dependent malic enzyme	2.81±0.03 2.78±0.22	0.03±0.01
Q9ULV4	COR1C	Coronin-1C	3.06±0.22	0.03±0.0
M0R116	MOR116	Sodium/potassium-transporting ATPase subunit alpha-3	3.13±0.67	0.05±0
P42224	STAT1	Signal transducer and activator of transcription 1-alpha/beta	2.62±0.09	0.05±0
P49721	PSB2	Proteasome subunit beta type-2	2.02±0.09 2.41±0.38	0.03±0.02
P11388	TOP2A	DNA topoisomerase 2-alpha	3.07±0.52	0.03±0.02 0±0
P06576	ATPB	ATP synthase subunit beta	3.13±0.53	0±0
Q9NTZ6	RBM12	RNA-binding protein 12	2.85±0.13	0.01±0.01
P12830	CDH1	Cadherin-1	2.85±0.15 2.30±0.10	0.01±0.01 0.04±0.01
P12830 P07355	ANXA2	Annexin A2	2.30±0.10 2.94±0.23	0.04±0.0
P07355 P27824-2	CALX	Isoform 2 of Calnexin		0±0 0±0
P27824-2 P49411	EFTU	Elongation factor Tu	2.81±0.04 3±0.31	0±0 0±0
Q96PK6	RBM14	-		
		RNA-binding protein 14 Mitochondrial 2-avaglutarate (malate carrier protein (Eragment)	3.89±0.53	0.01±0.01 0±0
I3L1P8	I3L1P8 ECHB	Mitochondrial 2-oxoglutarate/malate carrier protein (Fragment) Trifunctional enzyme subunit beta	2.89±0.11 3.27±0.51	0±0 0.01±0.02
P55084		i i nulleuonai enzvine subunit Deta	3.4/IU.51	0.01±0.04

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Accession	0		Fold change	P-value
NO.	Gene name	Protein name	(Mean±SD)	(Mean±SD)
Q6PIU2	NCEH1	Neutral cholesterol ester hydrolase 1	2.9±0.13	0±0
Q07960	RHG01	Rho GTPase-activating protein 1	2.72±0.39	0.01±0
P13797	PLST	Plastin-3	2.61±0.66	0±0
P31930	QCR1	Cytochrome b-c1 complex subunit 1	3.18±0.14	0±0
H0Y4R1	H0Y4R1	Inosine-5'-monophosphate dehydrogenase 2 (Fragment)	2.9±0.28	0±0
P07195	LDHB	L-lactate dehydrogenase B chain	2.59±0.77	0±0
075947	ATP5H	ATP synthase subunit d	3.63±0.71	0.02±0.01
P48047	ATPO	ATP synthase subunit O	3.65±0.73	0.04±0.01
Q9HDC9	APMAP	Adipocyte plasma membrane-associated protein	3.39±0.29	0±0
P23229	ITA6	Integrin alpha-6	3.16±0.08	0±0
P63104	1433Z	14-3-3 protein zeta/delta	2.71±0.77	0±0
043707	ACTN4	Alpha-actinin-4	6.61±1.84	0±0
Q8IVF2-3	AHNK2	Isoform 3 of Protein AHNAK2	3.94±0.84	0±0
P00492	HPRT	Hypoxanthine-guanine phosphoribosyltransferase	2.75±0.83	0±0
F5GZS6	F5GZS6	4F2 cell-surface antigen heavy chain	4.01±0.91	0±0
P22102	PUR2	Trifunctional purine biosynthetic protein adenosine-3	2.99±0.54	0±0
P08758	ANXA5	Annexin A5	2.85±0.73	0±0
Q13162	PRDX4	Peroxiredoxin-4	3.56±0.05	0.01±0.02
P13639	EF2	Elongation factor 2	2.98±0.38	0±0
J3KR97	J3KR97 EF1A1	Tubulin-specific chaperone D	2.99±0.16	0.01±0.01 0±0
P68104		Elongation factor 1-alpha 1	2.98±0.97	
075083	WDR1 K2C7	WD repeat-containing protein 1	3.35±0.54	0±0
P08729		Keratin, type II cytoskeletal 7 Microtubule-associated protein 1B	4.32±0.78	0±0
P46821	MAP1B TOP1	•	3.5±0.43 5.03±1.64	0±0
P11387 P30048	PRDX3	DNA topoisomerase 1 Thioredoxin-dependent peroxide reductase		0±0 0.02±0.01
P30048 P15311	EZRI	Ezrin	3.85±0.03 3.79±0.27	0.02±0.01 0±0
P15511 P14618	KPYM	Pyruvate kinase PKM	3.18±1.23	0±0 0±0
02TB90	HKDC1	Putative hexokinase HKDC1	4.31±0.36	0±0 0±0
P23528	COF1	Cofilin-1	4.51±0.50 3.55±0.71	0±0 0±0
Q8IXJ9	ASXL1	Putative Polycomb group protein ASXL1	4.85±1.13	0.04±0.01
P00338	LDHA	L-lactate dehydrogenase A chain	3.09±1.42	0±0
P17655	CAN2	Calpain-2 catalytic subunit	3.81±0.4	0±0
P18669	PGAM1	Phosphoglycerate mutase 1	3.08±1.48	0±0
P60174	TPIS	Triosephosphate isomerase	3.17±1.41	0±0
H0YIV4	H0YIV4	Nucleosome assembly protein 1-like 1 (Fragment)	3.85±0.45	0±0
P22314	UBA1	Ubiquitin-like modifier-activating enzyme 1	3.19±1.5	0±0
P23526	SAHH	Adenosylhomocysteinase	3.15±1.55	0±0
Q8WUM4	PDC6I	Programmed cell death 6-interacting protein	3.43±1.2	0±0
Q01469	FABP5	Fatty acid-binding protein, epidermal	3.41±1.24	0.01±0.01
015269	SPTC1	Serine palmitoyltransferase 1	4.05±0.5	0.02±0.01
P21333	FLNA	Filamin-A	3.92±1.11	0±0
P18206	VINC	Vinculin	3.62±1.53	0±0
P26641	EF1G	Elongation factor 1-gamma	4.23±0.98	0±0
P61758	PFD3	Prefoldin subunit 3	4.59±0.6	0±0
C9JFR7	C9JFR7	Cytochrome c (Fragment)	5.99±1.32	0±0
P15121	ALDR	Aldose reductase	3.84±1.92	0±0
Q92598	HS105	Heat shock protein 105 kDa	4.88±0.73	0±0
P37802	TAGL2	Transgelin-2	4.12±1.94	0±0
P60903	S10AA	Protein S100-A10	6.33±1.11	0.04 ± 0.01
P54709	AT1B3	Sodium/potassium-transporting ATPase subunit beta-3	5.15±0.7	0.01±0.01
P12429	ANXA3	Annexin A3	5.26±1.65	0±0
P17812	PYRG1	CTP synthase 1	5.27±2.07	0±0
Q14241	ELOA1	Transcription elongation factor B polypeptide 3	6.04±1.33	0.04±0.01
Q14019	COTL1	Coactosin-like protein	5.08±2.79	0±0
P05783	K1C18	Keratin, type I cytoskeletal 18	7.87±1.07	0±0
P04632	CPNS1	Calpain small subunit 1	6.43±0.96	0.03±0.01
P13489	RINI	Ribonuclease inhibitor	6.26±2.27	0.01±0.01
P29317	EPHA2	Ephrin type-A receptor 2	6.39±2.2	0±0
Q9BU23	LMF2	Lipase maturation factor 2	8.28±0.27	0±0
P55039	DRG2	Developmentally regulated GTP-binding protein 2	7.22±2.22	0.04±0
P09493-4	TPM1	Isoform 4 of Tropomyosin alpha-1 chain	10.42±0.2	0.02±0.01
P05787	K2C8	Keratin, type II cytoskeletal 8	11.78±1.3	0±0
060443	DFNA5	Non-syndromic hearing impairment protein 5	11.92±1.78	0.03±0.01
Q9Y2V2	CHSP1	Calcium-regulated heat stable protein 1	16.91±2.3	0.03±0.02

KEGG pathway analysis

KEGG analysis revealed 30 significant pathways with *P*<*0.05* (Fig. 3B.). The top ten pathways, including Ribosome biogenesis in eukaryotes (hsa03008), Spliceosome (hsa03040), mRNA surveillance pathway (hsa03015), Carbon metabolism (hsa01200), Steroid biosynthesis (hsa00100), Cysteine and methionine metabolism (hsa00270), Propanoate metabolism (hsa00640), Glycolysis/Gluconeogenesis (hsa00010), Biosynthesis of amino acids (hsa01230), and Cardiac muscle contraction (hsa04260), were displayed. Ribosome biogenesis in eukaryotes and spliceosome were the most significantly enriched pathways (Fig. 6).



Table 3. List of down-regulated p	proteins in TP-treated A549 cells
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Accession	Gene name	Protein name	Fold change	P-value
NO.	dene name	r toteni name	(Mean±SD)	(Mean±SD)
Q9H9Y2	RPF1	Ribosome production factor 1	0.06±0.01	0.02±0.01
02675	FIBB	Fibrinogen beta chain	0.32±0.07	0±0
96E29	MTEF3	Transcription termination factor 3	0.21±0.19	0.02±0.01
937268	FDFT	Squalene synthase	0.11±0.04	0±0
015213	WDR46	WD repeat-containing protein 46	0.13±0.04	0±0
02751	FINC	Fibronectin	0.14±0.05	0±0
000566	MPP10	U3 small nucleolar ribonucleoprotein protein MPP10	0.11±0	0±0
281Y37 213123	DHX37 RED	Probable ATP-dependent RNA helicase DHX37 Protein Red	0.21±0.15 0.12±0.01	0±0 0±0
213123 29Y3C1	NOP16	Nucleolar protein 16	0.07±0.06	0.01±0.01
07973	CP24A	1,25-dihydroxyvitamin D(3) 24-hydroxylase	0.1±0.02	0.01±0.01 0±0
)15397	K0020	Pumilio domain-containing protein KIAA0020	0.15±0.05	0±0
2TAY7	SMU1	WD40 repeat-containing protein SMU1	0.15±0.04	0±0
09601	HMOX1	Heme oxygenase 1	0.12±0.01	0±0
000767	ACOD	Acyl-CoA desaturase	0.17±0.06	0±0.01
9PB61	E9PB61	THO complex subunit 4	0.24±0.16	0±0
15050	RRS1	Ribosome biogenesis regulatory protein homolog	0.16±0.04	0±0
212860	CNTN1	Contactin-1	0.13±0.01	0±0
25VT52 213611	RPRD2 CSPG2	Regulation of nuclear pre-mRNA domain-containing protein 2 Versican core protein	0.08±0.07	0.03±0.02 0.01±0.01
9BVJ6	UT14A	U3 small nucleolar RNA-associated protein 14 homolog A	0.19±0.07 0.11±0.06	0.01±0.01 0±0
9BVI4	NOC4L	Nucleolar complex protein 4 homolog	0.14±0	0±0 0±0
75691	UTP20	Small subunit processome component 20 homolog	0.13±0.02	0±0
9H6R4	NOL6	Nucleolar protein 6	0.13±0.02	0±0
29NY93	DDX56	Probable ATP-dependent RNA helicase DDX56	0.1±0.07	0±0
00571	DDX3X	ATP-dependent RNA helicase DDX3X	0.32±0.07	0.02±0.02
296AQ6	PBIP1	Pre-B-cell leukemia transcription factor-interacting protein 1	0.24±0.13	0±0
9Y3A2	UTP11	Probable U3 small nucleolar RNA-associated protein 11	0.14±0.03	0.04±0.01
9J2Y9	C9J2Y9	DNA-directed RNA polymerase	0.13±0.04	0±0
213573	SNW1	SNW domain-containing protein 1	0.12±0.05	0.03±0.01
014776 22626	TCRG1 ROA2	Transcription elongation regulator 1 Heterogeneous nuclear ribonucleoproteins A2/B1	0.18±0.03	0±0 0±0
			0.18±0.03	0±0 0.04±0.01
014647	CHD2	Chromodomain-helicase-DNA-binding protein 2	0.15±0.03	0.0410.01
003701	CEBPZ	CCAAT/enhancer-binding protein zeta	0.15±0.03	0±0
9Y4C8	RBM19	Probable RNA-binding protein 19	0.17±0	0.02±0.02
2 5JTH9	RRP12	RRP12-like protein	0.14±0.05	0±0
Q9Y5J1	UTP18	U3 small nucleolar RNA-associated protein 18 homolog	0.17±0.01	0±0
213601	KRR1	KRR1 small subunit processome component homolog	0.23±0.07	0±0
9NSI2	F207A	Protein FAM207A	0.12±0.08	0.01±0.01
9BVP2	GNL3	Guanine nucleotide-binding protein-like 3	0.15±0.05	0.02±0.01
253EP0	FND3B	Fibronectin type III domain-containing protein 3B	0.27±0.11	0±0
)14692)96I24	BMS1 FUBP3	Ribosome biogenesis protein BMS1 homolog Far upstream element-binding protein 3	0.15±0.06 0.2±0.01	0±0 0±0
24928	RPB1	DNA-directed RNA polymerase II subunit RPB1	0.14±0.08	0±0 0±0
21920	RRP1	Ribosomal RNA processing protein 1 homolog A	0.17±0.04	0±0
29NV06	DCA13	DDB1- and CUL4-associated factor 13	0.3±0.15	0±0
076021	RL1D1	Ribosomal L1 domain-containing protein 1	0.12±0.11	0±0
C9JEU5	C9JEU5	Fibrinogen gamma chain	0.18±0.03	0±0
Q8TDD1	DDX54	ATP-dependent RNA helicase DDX54	0.22±0.02	0±0
Q14690	RRP5	Protein RRP5 homolog	0.17±0.05	0±0
076031	CLPX	ATP-dependent Clp protease ATP-binding subunit clpX-like	0.29±0.1	0±0
9BSC4	NOL10	Nucleolar protein 10	0.2±0.03	0±0
08TDN6	BRX1	Ribosome biogenesis protein BRX1 homolog	0.19±0.04	0.01±0.01
9ULH0 9NYH9	KDIS UTP6	Kinase D-interacting substrate of 220 kDa U3 small nucleolar RNA-associated protein 6 homolog	0.24±0.03 0.17±0.07	0±0 0.02±0.02
10Y714	H0Y714	U3 small nucleolar ribonucleoprotein protein IMP4 (Fragment)	0.18±0.07	0.02±0.02 0±0
		e e and a de com a contractor de la protein dan a (a raginetit)	0.1010.07	0±0
14866	HNRPL	Heterogeneous nuclear ribonucleoprotein L	0.2±0.04	
9UNQ2	DIM1	Probable dimethyladenosine transferase	0.14±0.13	0.04±0
1KMD3	HNRL2	Heterogeneous nuclear ribonucleoprotein U-like protein 2	0.28±0.06	0±0
42696	RBM34	RNA-binding protein 34	0.23±0.01	0.01±0.02
9H0A0	NAT10	N-acetyltransferase 10	0.22±0.02	0±0
28IV08	PLD3	Phospholipase D3	0.35±0.16	0.01±0.02
9BZE4	NOG1	Nucleolar GTP-binding protein 1	0.17±0.09	0±0
38919 95478	EIF4A3 NSA2	Eukaryotic initiation factor 4A-III Ribosome biogenesis protein NSA2 homolog	0.16±0.12 0.17±0.1	0±0 0±0
95478 948449	NSAZ ERG7	Lanosterol synthase	0.17±0.1 0.28±0.04	0±0 0±0
31327	CPSM	Carbamoyl-phosphate synthase [ammonia]	0.33±0.11	0±0 0±0
16615	AT2A2	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	0.24±0.01	0±0 0±0
10253	LYAG	Lysosomal alpha-glucosidase	0.25±0	0.01±0.01
6DKI1	RL7L	60S ribosomal protein L7-like 1	0.22±0.05	0.04±0.01
9H7B2	RPF2	Ribosome production factor 2 homolog	0.24±0.01	0.05±0
8NEJ9	NGDN	Neuroguidin	0.22±0.05	0±0
201123338	DX39B	Spliceosome RNA helicase DDX39B	0.17±0.12	0.02±0.02
25QJE6	TDIF2	Deoxynucleotidyltransferase terminal-interacting protein 2	0.17±0.12	0.04±0.01
17C2Q8	H7C2Q8	EBNA1 binding protein 2, isoform CRA_d	0.2±0.08	0±0
41223	BUD31	Protein BUD31 homolog	0.16±0.14	0.03±0.01
9NRX1	PNO1	RNA-binding protein PN01	0.32±0.08	0±0
42205	SK2L2	Superkiller viralicidic activity 2-like 2	0.2±0.09	0±0
	NOL7	Nucleolar protein 7	0.21±0.07	0±0
9UMY1				
9UMY1 8IY81	SPB1	pre-rRNA processing protein FTSJ3	0.23±0.05	0±0
9UMY1 8IY81 14137	SPB1 BOP1	Ribosome biogenesis protein BOP1	0.23±0.05	0±0
242285 29UMY1 28IY81 214137 296GQ7 206865	SPB1			

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Li et al.: Proteomics Analysis of Triptolide Targets in A549 Cells

No. (Main SD) (Mai	Accession			Fold change	P-value
qc2948 FUIPZ Pur uptasam element halding protein 2 qc22.0.1 0.10 q6943 PKN Poly(A)-polet iterminant Qc29.0.3 Qc20.0.3	NO.	Gene name	Protein name	(Mean±SD)	(Mean±SD)
09553 PANN Poly(A)-specific rhounckais PANN 0.24.0.12 0.94.0 0[14]44 CKN13 Gaanne nuckenize-binding provin in sunit alpha-13 0.399.0.12 0.011.0.0 0[34]44 CKN13 Gaanne nuckenize-binding provin in sunit alpha-13 0.399.0.12 0.011.0.0 0[34]44 CKN13 General transcription factor I-I 0.389.0.11 0.011.0.0 0[34]43 GTS21 Canton nuckenize-binding provin in 3 0.24.0.1.5 0.011.0.0 0[31]23 NGG2 Nuckeenic (TP-binding provin in 2 0.24.0.1.5 0.011.0.0 0[31]23 NGG2 Nuckeenic (TP-binding provin in 2 0.24.0.1.6 0.011.0.0 0[31]23 NGG2 Nuckeenic (TP-binding provin in 2 0.24.0.1.6 0.011.0.0 0[31]23 R107 UI samit nucker ribonuckoprotein 7 kba 0.24.0.0 0.024.0.0 0[31]23 R107 Peptdyloptyl somerosa schamit and WD provine in 2 0.24.0.0 0.024.0.0 0[31]24 GR Granulina Granulina 0.34.0.0 0.024.0.0 0[31]35 R10 Feropsteneme elsens thinding provin 1<	043172	PRP4	U4/U6 small nuclear ribonucleoprotein Prp4	0.24±0.07	0±0
00-119 CDC73 Deraffrommin 0.27:0.03 0.01 0.01 073316 NO143 Gamme nucleotic-holling proteins subunit alpha-13 0.37:0.13 0.01 <td< td=""><td></td><td></td><td></td><td></td><td></td></td<>					
0.14.34 CNA13 Gamine nucleotide-binding protein subunit alphe 1-3 0.29.0.12 0.010 0.77316 NO14 Nucleokar protein 14 0.17.0.13 0.01 0.77317 Construction Construction 0.39.0.13 0.01 0.01 0.77317 Construction Construction 0.39.0.13 0.39.0.13 0.39.0.13 0.39.0.13 0.39.0.13 0.39.0.13 0.39.0.13 0.39.0.13 0.39.0.13 0.39.0.13 0.39.0.13 0.39.0.13 0.39.0.13 0.39.0.0 0.49.0.12 0.22.0.10 0.03.0.0 0.39.0.0 0.22.0.13 0.03.0.0 0.03					
TP3316 NOP14 Nuckokar protein 14 0.17.0.13 0.90 0792144 DOSZ2 Dvabak 274-beyendent RNA helicase DDSZ 0.19.0.10 0.01 0.01 0793147 CTF21 General transcription factor 1-1 0.38.0.11 0.40 0798173 BEV23 BWABA DVADAK 0.22.0.15 0.42.0.15 0798131 RAGO Heterogeneous nucker rhomuckeprotein 2 0.22.0.16 0.04.0.10 0708131 RAGO Heterogeneous nucker rhomuckeprotein 2 0.22.0.16 0.01.0.01 070813 RAGO Heterogeneous nucker rhomuckeprotein 2 0.22.0.10 0.02.0.01 070815 SF1 UT Disposition remotein 40 0.22.0.10 0.02.0.01 070815 SF1 Paputdybroylis comercase chomina null by repact-chomining protein 1 0.23.0.0.0 0.02.0.01 079814 DC110.01 DC210.01 0.02.0.01 0.02.0.01 079814 DC210.01 DC210.01 0.02.0.01 0.02.0.01 079817 Paputdybroylis comercase chomina null by repacchominin 40 0.22.0.0.1 0.02.0.0					
P73847 GTP21 General inserption factor II-1 0.3810.11 0.10 G3V1C3 GAV1C3 Approxis inhibitor 5 0.21.01.31 0.40 QMV13 RBM28 RRM4-binding protein 28 0.22.01.51 0.49 QM3313 NOC2 Approxis Inhibitor 5 0.22.01.51 0.49 QM3313 ROAD Heterogeneous nucker ribonuckoprotein 70 RDa 0.22.01.12 0.01.40 QM3613 RU17 U small nuckar ribonuckoprotein 70 RDa 0.32.00.01 0.01.01 PORD55 SEP1 Peptidyloryli Somerase domain and Win Speet-consining protein 1 0.21.01.01 0.02.00.02 PS6571 LICH Lysosomal acid Ipase/cholesteryl ester hydrobase 0.19.01.10 0.02 QM3624 PUBP1 Fur upsters nuclear ribonuckoprotein 20 0.31.01.10 0.01 QM3705 RRM2-9 Matrin 3 0.23.01.01 0.01 0.02 QM3705 RRM2-9 Matrin 3 0.23.01.01 0.01.00.01 0.02.00.01 QM3705 RRM2-9 Matrin 3 0.02.00.01 0.02.00.01 0.02.00.01 <td></td> <td></td> <td>Nucleolar protein 14</td> <td></td> <td></td>			Nucleolar protein 14		
G3V1.3 G3V1.3 Approtosi inhibitor 5 0.21.0.1.3 0.10 Q4W0V13 RMM28 RNA-binding protein 28 0.22-0.0.8 6.10 Q13823 NOC2 Amphoterm-induced protein 20 0.23.0.16 0.40 Q13825 NOC2 Amphoterm-induced protein 70 kbn 0.23.0.04 0.01:0.01 Q13825 NOC2 Protein LAP2 0.23.0.04 0.02:0.00 Q19055 SSF1 Suppressor 65W141 homoly 0.44.0.12 0.02:0.01 Q27079 GRN Protein/poly isomerasor 65W141 homoly 0.34.0.03 0.02:0.01 Q27079 GRN Expressor 63W141 homoly 0.34.0.03 0.02 Q480079 ABMAP9 Marina 3 0.23.0.01 0.02 Q480079 ABMAP9 Marina 3 0.23.0.01 0.02 Q43355 PEP73 HARF Hetrogeneous nucker ribouncekoprotein F 0.22.6.0.1 0.02 Q43355 PEP73 U4 (Jo Samin uncker ribouncekoprotein F 0.23.26.00 0.02 Q43355 PEP73 U4 (Jo Saminuncesuppressor canddatter stetr					0.01±0.01
QPMV13 RBM28 RXA-binding protein 28 Q.220.15 0.40 QB65[2 MGCQ Angobachar CTP-binding protein 2 Q.250.16 0.41 Q13151 ROAD Nuckobar CTP-binding protein 2 Q.250.16 0.41 QPM055 RU17 U1 small nuckar ribonuckoprotein 70 kDa 0.324.03 0.01100 PS1777 FS17777 Psptidyiproyi Isomerase domain and WD repeat-containing protein 1 0.214.01 0.012.00 QPM055 SSF1 Suproser of SW41 Jonobay 0.319.03 0.032.00.01 QPM056 BRD4 Containing protein 1 0.214.01 0.021.00 QPM057 SSF1 Suprosen and acto containing protein 4 0.381.00 0.021.00 QPM389 BRD4 Promodemain-containing protein 4 0.324.01 0.021.00 QPM389 RPF15 WRFF Herogenous nuckar ribonuckoprotein Trp3 0.324.00 0.0224.00 QPM389 RPF15 US Protein 0.324.00 0.0224.00 QPM389 RPF15 US Protein 0.324.00 0.021.00 QPM380 <					
Qé652 AMGO2 Amphoterin-indiced protein 2 0.25:0.08 0:10 Q13823 NOG2 Nuckour GTP-binding protein 2 0.22:0.13 0.04:0.01 Q13811 ROAD Heterageneous nucker ribonuckeprotein 70 MDa 0.32:0.03 0.04:0.01 Q10811 RUIT U. sull nucker ribonuckeprotein 70 MDa 0.32:0.03 0.03:0.01 P17797 FP17797 Peptidylproly isomerase domain and WD repeat-containing protein 1 0.21:0.01 0.02:0.02 P38571 LICH Lysosomal acid lpase/cholestery lester hydrobase 0.19:0.18 0.02:0.02 Q64684 FURP1 Fur uptrase demensibiling protein 1 0.32:0.03 0.02:0.02 Q64885 BRUKP Bromochamia- containing protein 20 0.38:0.01 0.02:0.01 Q75543 US2:0 US snall unckers ribonuckeprotein 200 KD helicase 0.38:0.01 0.02:0.01 Q75748 US2:0 US snall unckers ribonuckeprotein 200 KD helicase 0.35:0.01 0.02:0.01 Q75743 US2:0 US snall unckers ribonuckeprotein 1 0.42:0.00 0.02:0.01 Q75743 US2:0 US snallu un					
Q13151 ROA0 Hetrogeneous nucker ribonickoprotein 70 kDa Q22:0.13 0.04:0.01 P06671 RU17 U's mall nucker ribonickoprotein 70 kDa Q.34:0.03 0.04:0.01 P07055 SSF1 Peptidytroph isomerase due Grandine Q.34:0.03 0.03:0.01 P07075 FSG3M Peptidytroph isomerase due Grandine Q.34:0.03 0.03:0.03 P05654 PCG3M Peptidytroph isomerase due Grandine protein 1 Q.24:0.10 0.02:0.02 P056571 LICH Lycosomal actil (pase/hobster) elser hydrobase 0.19:0.10 0.02:0.02 P052577 HRPF Hetrogeneous nucker rhonuckeprotein F 0.24:0.01 0.01 P05257 HRPF Hetrogeneous nucker rhonuckeprotein F 0.24:0.01 0.01 P05257 HRPF Hetrogeneous nucker rhonuckeprotein F 0.22:0.01 0.02:0.01 Q132:0.3 TIFIR Transcription intermediary factor 1-beta 0.22:0.01 0.02:0.01 Q132:0.4 Coll division cyck and apotosis regulator protein 0.22:0.01 0.02:0.01 Q13:0.1 Cuck and apotosis regulator protein 0.22:0.01	Q86SJ2		Amphoterin-induced protein 2		
QösGT1 LAP2 Protein LAP2 0.28e.0.4 0.01±001 QWRQ25 SSF1 Suppressor of SW4 1 homokg 0.440.12 0.01±001 PSH777 Peptdylprobl Someras domain and WD repeat-containing protein 0.28e.0.4 0.02±0.01 PSH771 FUT Peptdylprobl Someras domain and WD repeat-containing protein 0.310.01 0.02±0.01 PSH571 LICH Fur upstream containing protein 0.23±0.14 0.02±0.01 AMMP9 AdMMP9 Marrin-3 0.23±0.01 0.02±0.01 OR0885 BR194 Bromochamia-containcoptrotein Pry3 0.22±0.03 0.e0 OV3395 PRFF3 U4/UG Start Inducker prioon protein 0.24±0.01 0.24±0.01 OY5443 U52.01 U53 mall inducker ribounckeportein 200 Ub-helinose 0.24±0.01 0.24±0.01 OY5443 U52.01 U4/UG U55 mall inducker ribounckeportein 1 0.24±0.01 0.24±0.01 OY5543 CCARL Cell division operasit radius region protein 1 0.24±0.01 0.01±0.01 QWNL3 CCARL Cell division operasit radius region radiu radius region radiu radius region radiu radiu r					
P00621 BU17 U1 small nuckar ribonuckoprotein 79 kba 0.340.03 0.0220.01 P5H777 P5H777 Peptidybyroly isomerase domain and WD repact containing protein 1 0.279.0.4 0.0116.00 Q40625 SSF1 Suppressor 55W14 1.031.0.3 0.0220.01 Q4644 FUBP1 Far upstream chement-binding protein 1 0.214.0.17 0.024.0.01 Q4644 FUBP1 Far upstream chement-binding protein 1 0.224.0.01 0.022.0.01 Q40809 ABR49 Fromodynamic-comming protein 4 0.334.0.03 0.022.0.01 Q43395 FRP13 U4/1/05 small nuckar ribonuckoprotein Pr3 0.324.0.03 0.022.0.01 Q43395 FRP13 U4/1/105 small nuckar ribonuckoprotein 200 Rab helicase 0.338.0.05 0.022.0.01 Q43205 FRP13 The appressor and/Adatir region gene 2 protein 0.224.0.17 0.60 Q43215 GCR21 Gliona tumor suppressor and/Adatir region gene 2 protein 0.232.0.18 0.022.0.01 Q43245 GCR21 Calmin-1 0.424.0.02 0.0116.0.0 Q43245 GCR21 Calmin-1					
FSH77 Peptidybyrolj somerise domain and WD repeat-containing protein 1 0.29e.0.4 0.022e.0.1 P28799 GRN Granulins 0.310.3 0.032e.0.1 P28791 LiCH Lysoomal acil ipase / chosteryl ester hydrokse 0.21e.0.1 0.021e.0.1 ABM/M* ABM/M* Barin-3 0.23e.0.14 0.021e.0.1 0.021e.0.1 P38571 LiCH Lysoomal acil ipase / chosteryl ester hydrokse 0.22e.0.11 0.010 Q43395 PRPF3 U4/U6 small nucker ribonuckoprotein PD3 0.32e.0.03 0.022e.0.01 Q75643 U520 U5 small nucker ribonuckoprotein 200 R0a helicase 0.38e.0.05 0.022e.0.01 Q75743 U520 U5 small nucker ribonuckoprotein 200 R0a helicase 0.35e.0.01 0.011.0.01 Q75743 U520 U5 small nucker ribonuckoprotein 200 R0a helicase 0.35e.0.01 0.011.0.01 Q75743 U570 CGinaa tumor suppressor candidate region gene 2 protein 2.22e.0.18 0.021.0.01 Q75743 U571 DAZ-associated protein 1 0.32e.0.06 0.011.0.01 Q10570 CFFFI CAvage and					
P28799 GRN Granulins Granulins 0.340.03 0.340.03 0.0340.01 P368571 LICH Lysosonal acid lipase/choksteryl estr hydrokse 0.190.14 0.024002 ABMKP9 Martin ⁻³ 0.23.014 0.00 P38571 LICH Lysosonal acid lipase/choksteryl estr hydrokse 0.190.14 0.02 P052597 INNPF Heterogeneous nucker rhounckoprotein F 0.2240.01 0.03 P075493 RRP15 W/f/to smoother rhounckoprotein 200 Kb helicase 0.3840.05 0.0210.01 Q97543 US20 US small nucker rhounckoprotein 200 Kb helicase 0.2240.01 0.0210.01 Q38161 CULL Collin-1 0.2240.01 0.0210.01 Q38161 CULL Collin-1 0.2240.01 0.011.00 Q43260 SWIT1 CARNE CARNE 0.0334.00 0.011.00 Q43261 SWIT1 CARNE DAParescharde protein 1 0.2340.00 0.011.00 Q4230 SY331 SY311 Sy110.00 DAParescharde protein 1 0.0340.01 0.0340				0.4±0.12	0.01±0.01
096.64: FUBP1 Far upstream element-binding protein 1 0.2140.17 0.04 P38571 LICH Lysosomal acil lipasc/chockrey elster hydroisze 0.190.18 0.022002 060885 BRD4 Bromokamia- containing protein 4 0.380.007 0.60 073305 FRIP3 U/L/LOS mall nucker rhoouckeprotein Fry3 0.224.003 0.61 073305 FRIP3 U/L/LOS mall nucker rhoouckeprotein Fry3 0.224.017 0.61 073305 FRIP3 U/L/LOS mall nucker rhoouckeprotein Fry3 0.224.017 0.61 073305 FRIP3 Castanti Fry1 0.224.011 0.024.01 0.024.01 073305 FRIP3 Castanti Fry1 0.224.017 0.014.00 0.024.01 073305 SNUT1 U/L/LOS tri-snRNP-associated protein 1 0.234.004 0.014.00 073533 ST312 Splicing factor TMMC1 0.324.006 0.60 075533 ST312 Splicing factor TMMC1 0.324.006 0.61 073533 ST312 Splicing factor TMMC1 0.324.00 0.610.00					
P38571 LICH Lysosonni acid lipsav/chokstery/ ester hydrokse 0.19-0.18 0.02:002 ABMXP9 ABMXP9 Martin-3 0.23.00.14 0.60 060885 BRD4 Bromodonain-containing protein 4 0.3380.07 0.69 075395 PRPT5 U4/US small nucker ribonuckeprotein Prp3 0.32.00.31 0.62 075443 U520 US small nucker ribonuckeprotein 200.02.04.01.1 0.02:00.01 0.02:00.01 031263 LIFLB Cali division cyck and apoptosis regulator protein 0.45:00.01 0.02:00.01 043290 SNITT U4/US UF sinNNP-ascicated protein 0.33:00.01 0.01:00.01 043306 CUENT Colina turkitor bir sinNN-ascicated protein 1 0.33:00.01 0.01:00.01 043501 TIDICI Competal Ascettybalcosaminytransferase 0.33:00.01 0.01:00.01 043502 DAZ2-10 DAZ-associated protein 1 0.32:00.06 0:00 043502 DZ240.02 Berlon 0.01:00.01 DZ3:00.01 0.01:00.01 043503 DZ320.06 0.00:01:00.01 DZ3:20.06 0:					
ABMKP9 ABMKP9 Marin-3 C.236.0.07 C.237.0.14 C.64 050885 BRD4 Bronodmain-containing protein 4 C.238.0.07 C.60 043395 PRPF3 U4/US mail nucker tribonuckoprotein FP3 C.236.0.13 0.60 075643 U520 US mail nucker tribonuckoprotein Prot C.286.0.15 0.60 075643 US20 US mail nucker tribonuckoprotein Prot 0.240.0.15 0.60 081X12 CCAR1 Cell division cycle and appolics regulator protein 0.224.0.18 0.024.0.01 091X12 CCAR1 Cell division cycle and appolic protein 1 0.236.0.11 0.024.0.01 091X13 CCAR1 Communor suppressor candidate region gene 2 protein 0.224.0.16 0.014.0.01 091X13 FD01 Complex Lassemply factor TMMC11 0.324.0.06 0.014.0.01 090757 CPSF1 Ceavage and polyachystion specifici factor rabunit 1 0.324.0.07 0.024.0.01 012323 SF381 Splicing factor 276 sk bla shunit 1 0.324.0.07 0.040 012323 SF381 Splicing factor 276 sk bla shunit 1					0.02±0.02
P52597 HNRF Heterogeneous nuckar ribonuckoprotein F 0.25e.0.3 0.49 Q43395 RRPF3 U4/U6 small nuckar ribonuckoprotein Prp3 0.22t.0.03 0.49 Q75643 U520 U5 small nuckar ribonuckoprotein 200 Kbh helicase 0.28t.0.05 0.69 Q3263 TTF1B Transcription intermediary factor 1-ben 0.22t.0.13 0.040 Q8XL2 CCAR1 Cell division cycle and appoints regulator protein 0.24t.0.18 0.021.00 Q33616 CU11 U4/U6.05 ris.nRV-associated protein 1 0.23t.00 0.011.00 Q31616 CU11 Comptoh assembly to sociated protein 1 0.32t.00 0.011.00 Q96FPS DAZ71 Ceavage and DAZ-associated protein 1 0.32t.00 0.011.00 Q35333 SF381 Splicing factor 278 shunit 1 0.42t.00.60 0.012.00 Q13533 SF381 Splicing factor 278 shunit 1 0.43t.00.70 0.014.00 Q13543 G7814 Kas GTPase-activating protein-binking protein 1 0.33t.00.70 0.014.00 Q13630 G3814 Asylolifases E 0.43t.00.70					
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097393 RRP15 IRRP15-like protein 0.281.00.5 0.024.001 075643 US20 US small nuckear ribonuckoprotein 200 kbh helicase 0.2381.00.5 0.040 081X12 CCAR1 Cell division cyck and appotrosis regulator rubein 0.224.01.1 0.014.00 09X7M5 GSCR2 Glioma tumor suppressor candidate region gene 2 protein 0.224.01.1 0.014.00 013206 CUL1 Cullin-1 0.334.00 0.014.00 013616 CUL1 Cullin-1 0.324.00 0.014.00 010570 CPFG1 Ckavaga end polyadanykation specificity factor subunit 1 0.324.006 0.04 0105753 SF381 Splicing factor 38 subunit 0.324.006 0.040 013523 FF142 Splicing factor 38 subunit 0.248.013 0.031.001 013523 FF142 Arystaffacase E 0.436.007 0.040 013630 TF7485 Heterogeneous nucker ribonuckoprotein 1.1ke protein 1 0.384.001 0.018.001 013645 CF31A Lanosterol 14-alpha damethylse 0.246.019 0.018.001					
075643 US 20 US small nuckar ribonucleoprotein 200 kDa helicase 0.340.05 0.40 Q13263 TIFIB Transcription intermediary factor 1-beta 0.2240.17 0.40 Q9NZM5 GSCR2 Clionat imors suppressor candidate region gene 2 protein 1 0.4240.08 0.02240.11 Q33616 CULL1 Cullin-1 0.3340.04 0.01140 Q9NZM5 FRW1J3 Polypeptick N-acetygalactosaminytransferase 0.3340.01 0.01140 Q9NPLB TIDC1 Complex 1 assembly factor TIMMDL1 0.3240.06 0.60 Q9NPLB DAZF Splicing factor 38 subunit 1 0.3240.06 0.60 Q755333 SF381 Splicing factor 38 subunit 1 0.3240.06 0.010.01 Q13264 CL2AF1 Splicing factor 38 subunit 1 0.3240.06 0.011.00 Q13283 G3BP1 Ras GTPse-activating protein 1 0.3340.07 0.014.01 Q14545 Splicing factor 38 subunit 1 0.3340.07 0.014.01 Q15459 SF3A1 Protein mago protein 1-ding protein 1 0.3340.07 0.0340.01 Q1454					
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QPNZM5 GSCR2 Ginan tumor suppressor candidate region gene 2 protein 0.2240.11 0.0140.01 Q13616 CUL1 Cullin-1 0.3340.04 0.0140.01 Q13616 CUL1 Cullin-1 0.3340.04 0.0140.01 Q18701 Complex1 assembly factor TIMMDC1 0.3240.07 0.0140.02 Q10870 CFSF1 Ckavage and polyadenytation specificity factor subunit 1 0.3240.06 0.04 Q13523 S73B1 Splicing factor 38 subunit 1 0.4240.08 0.0340.01 Q13523 FFR48 Serrine/thronoine-protein-inkinase RPA Homobg 0.3340.01 0.0340.01 Q13523 G28P14 Ras GTPase-activating protein-inding protein 1 0.3340.07 0.04 Q13523 G28P14 Ras GTPase-activating protein-inding protein 1 0.3340.01 0.04 Q13624 FF7418 Hetrogeneous nuckar ribonuckeprotein 1-1 0.3340.01 0.04 Q14545 SF3A1 Splicing factor 3 subunit 1 0.3440.01 0.0340.01 Q145459 SF3A1 Splicing factor 3 subunit 1 0.3440.01 0.04	Q13263	TIF1B	Transcription intermediary factor 1-beta		0±0
043290 SNUT1 U4/U6.U5 m-snRNP-associated protein 1 0.236.04 0.01±0 Q13616 CULL Cullin-1 0.332.0.04 0.01±0 QVNPL8 FRSVUJ3 Polypeptic N-acetykalectosaminybransferase 0.351.0.01 0.01±0.01 QVNPL8 TIDC1 Complex 1 assembly factor TMMDC11 0.322.0.06 0.04 Q96EP5 DAZP1 Cleavage and polyadenylation specificity factor subunit 1 0.322.0.06 0.04 P26368 U2AP2 Splicing factor 24.6 is buouti 0.238.0.01 0.033.0.01 Q13533 SP381 Splicing factor 24.6 is buouti 0.238.0.0 0.033.0.01 Q136353 SP814 Ras GTPase-activating protein-binding protein 1 0.338.0.0 0.033.0.01 Q136353 SP341 Ras GTPase-activating protein-binding protein 1 0.348.0.0 0.00 Q134549 SP341A Splicing factor 24.8 is buoit 1 0.414.0.0 0.033.0.01 Q14545 SP341 Splicing factor 34.subunit 1 0.414.0.0 0.014.0.0 Q14444 G05 ribosomal protein 1.4 0.340.0 0.044.0.0 0.01					
Q13616 CUL1 Cullin-1 0.33±0.04 0.01±0.01 QNPLB TIDC1 Complex lassembly factor TIMMDC1 0.33±0.07 0.01±0.01 QNPLB TIDC1 Complex lassembly factor TIMMDC1 0.32±0.06 0±0 QNEPES DAZP1 DAZ-associated protein 1 0.32±0.06 0±0 QNEPES DAZP1 DAZ-associated protein 1 0.42±0.08 0±0.01 PS6368 UZAF2 Splicing factor 38 subunit 1 0.42±0.08 0±0.01±0.01 Q13523 PRP4B Serie (Presonier protein kinase PRP4 homobg 0.33±0.07 0±0 Q13283 G3BP1 Ras GTPase-activating protein-binding protein 1 0.33±0.07 0±0 Q145459 SF3A1 Lanosterol 14-alpha demethylase 0.24±0.19 0.033±0.01 Q145459 SF3A1 Splicing factor 34 subunit 1 0.34±0.07 0±0 Q46472 MGN2 Protein mage nashi homokg 2 0.24±0.10 0.03±0.01 Q46472 MGN2 Protein mage nashi homokg 2 0.24±0.10 0.03±0.01 Q464744 CAPR1 C					
F8VUJ3 F9VUJ3 Polypeptide N-acetykgalectosaminyltransferase 0.3540.01 0.014.002 Q9NPL8 TIDC1 Complex lassembly factor TIMMC1 0.3240.06 0.40 Q9GEPES DAZ-associated protein 1 0.3240.06 0.40 Q9GEPES DAZ-associated protein 1 0.3240.06 0.40 Q9GEPES DAZ-associated protein 1 0.3240.06 0.40 Q13523 PRP4B Splicing factor V2AF 65 KDa subunit 1 0.4240.08 0.40.01 Q13523 PRP4B Serine/threonine-protein kinase PRP4 homolog 0.3340.01 0.030.01 Q14583 G3BP1 Ras GT2as-cativating protein 1 0.416.07 0.40 Q15459 SF3A1 Lanosterol 14-sipha demethysize 0.2440.10 0.016.00 Q15459 SF3A H4 605 ribosomal protein L4 0.3340.01 0.60 Q1452 EIF3A Eukaryotic transkiton initiation factor 3 subunit 1 0.416.07 0.61 Q14547 MGN2 Protein mago nashi homokg 2 0.2461.0 0.01 0.0340.01 0.01 0.01 0.0340.01					0.0 0.0 -
Q10570 CPSF1 Cleavage and polyadenylation specificity factor subunit 1 0.3240.06 0-00 Q96EP5 DAZ- Splicing factor 3B subunit 1 0.3240.06 0-00 Q15533 SF3B1 Splicing factor 2B subunit 1 0.2440.08 0.010.10 Q13523 PRP4B Serine/threonine-protein kinase PRP4 homolog 0.3340.01 0.0340.01 Q13283 G3BP1 Ras GTPase-activating protein 1 0.4140.07 0-60 Q15489 SF3A1 Lanosterol 14-4.0jha damethylase 0.2440.19 0.0340.01 Q15459 SF3A1 Lanosterol 14-ajha damethylase 0.2440.19 0.0340.01 0-60 Q15459 SF3A1 Elakaryotic transholm initiation factor 3 subunit 1 0.4160.03 0-61 Q14152 EIF3A Eukaryotic transholm okg 2 0.2440.10 0-61 Q15459 RL4 605 ribosomal protein L4 0.3340.07 0-60 Q1444 CAPRI Caprin-1 0.3340.01 0-60 Q1444 CAPRI Caprin-1 0.3340.01 0-60 Q2900 R	F8VUJ3	F8VUJ3	Polypeptide N-acetylgalactosaminyltransferase		0.01±0.02
096EP5 DA2P1 DA2-associated protein 1 0.3240.06 0-01 075533 SF3B1 Splicing factor 3B subunit 1 0.4240.00 0.0140.01 075533 SF3B1 Splicing factor 3B subunit 1 0.4240.00 0.0140.01 071532 PRP4B Serincip factor 3B subunit 1 0.3240.01 0.0336.00 071533 G3BP1 Ras GTPas-activity ing protein-hinding protein 1 0.3340.07 0.40 071533 G3BP1 Ras GTPas-activity ing protein binding protein 1 0.3410.07 0.40 0715459 SF3A1 Lanosterol 14-alpha demethylase 0.03410.07 0.60 07647 MGN2 Protein mago nashi homokg 2 0.0240.02 0.0110.01 076578 RL4 605 ribosonal protein L4 0.3340.07 0.80 075578 RL4 Heterogeneous nuckar ribonuckoprotein L4 0.3340.1 0.013-00.02 075448 MED24 Mediator of RNA polymerase It transcription subunit 24 0.3640.06 0.0340.01 024404 CAPRI Caprin 1 Restormal caprin 1 0.3440.04 0.01 <td></td> <td></td> <td></td> <td></td> <td>0.01±0.01</td>					0.01±0.01
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P26366 U2AF2 Splicing facTor U2AF 65 kDa subunit 0.286.012 0.0110.01 PRF4B Serine/threonine-protein kinase RPR homobg 0.35.003 0.0310.01 PG1323 G3BP1 Ras GTPase-activating protein-binding protein 1 0.334.007 0.02 Q13243 G3BP1 Ras GTPase-activating protein-binding protein 1 0.334.001 0.03 Q15459 SFSA1 Splicing factor 3A subunit 1 0.414.003 0.02 Q14452 KIN2 Protein mago nash homolog 2 0.244.0.2 0.011.001 Q14545 RL4 605 ribosomal protein L4 0.348.001 0.00 Q65578 RL4 Heterogeneous nuckar ribonuckoprotein L-like 0.348.001 0.010.00 Q14444 CAPR1 Caprin-1 0.334.01 0.011.00 Q1444 CAPR1 Caprin-1 0.348.00 0.034.01 Q03341 VIGLN Wediator of RNA polytemersel I transcription subunit 24 0.366.06 0.034.01 Q0341 VIGLN Wediator of RNA polytemersel transcription subunit 3 0.414.00 0.014.02 Q9NY64					
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Table 4: The 50 apoptosis-associated proteins of enrichment analysis

Protein-protein interaction network analysis To further examine the comprehensive information obtained from the identified protein data, the PPI network was analyzed. The network model was generated using the Cytoscape web application based on information gained

in up to 4 levels of

sis: fold-change of gene/protein

pression, protein-

tions, KEGG path-

and biological pro-

A merged network is shown in Fig. 7. Again, PPI analysis identified ribosome biogenesis in eukaryotes and the spliceosome as the

analy-

interac-

enrichment

enrichment.

ex-

functional

protein

way

cess

Accession Fold chang Gene name Protein name	
NO. Protein name (Mean±SD) (Mean±SD)
P35232 PHB Prohibitin 2.61±0.4	0.01±0.02
013464 ROCK1 Rho-associated protein kinase 1 2.75±0.61	0±0
P30101 PDIA3 Protein disulfide-isomerase A3 2.15±0.2	0±0
09BXJ9 NAA15 N-alpha-acetyltransferase 15, NatA auxiliary subunit 2.1±0.1	0±0
P09874 PARP1 Poly [ADP-ribose] polymerase 1 2.48±0.46	0±0.01
P21796 VDAC1 Voltage-dependent anion-selective channel protein 1 3.01±0.17	0±0
007021 C10BP Complement component 1 0 subcomponent-binding protein 2.52±0.47	0.03±0.01
P45880 VDAC2 Voltage-dependent anion-selective channel protein 2 2.93±0.69	0±0
P07237 PDIA1 Protein disulfide-isomerase 2.54±0.23	0±0
P05556 ITB1 Integrin beta-1 2.46±0.11	0±0
P48506 GSH1 Glutamatecysteine ligase catalytic subunit 2.49±0.03	0.01±0.01
P42224 STAT1 Signal transducer and activator of transcription 1-alpha/beta 2.62±0.09	0±0
P49721 PSB2 Proteasome subunit beta type-2 2.41±0.38	0.03±0.02
P11388 TOP2A DNA topoisomerase 2-alpha 3.07±0.52	0±0
P12830 CDH1 Cadherin-1 2.30±0.10	0.04±0.01
P23229 ITA6 Integrin alpha-6 3.16±0.08	0±0
P63104 1433Z 14-3-3 protein zeta/delta 2.71±0.77	0±0
043707 ACTN4 Apha-actinin-4 6.61±1.84	0±0
P08758 ANXA5 Annexin A5 2.85±0.73	0±0
P08729 K2C7 Keratin, type II cytoskeletal 7 4.32±0.78	0±0
P11387 TOP1 DNA topoisomerase 1 5.03±1.64	0±0
P30048 PRDX3 Thioredoxin-dependent peroxide reductase 3.85±0.03	0.02±0.01
P14618 KPYM Pvruvate kinase PKM 3.18±1.23	0±0
P23528 COF1 Cofilin-1 3.55±0.71	0±0
P00338 LDHA L-lactate dehydrogenase A chain 3.09±1.42	0±0
Q8WUM4 PDC6I Programmed cell death 6-interacting protein 3.43±1.2	0±0
P21333 FLNA Filamin-A 3.92±1.11	0±0
P15121 ALDR Aklose reductase 3.84±1.92	0±0
Q92598 HS105 Heat shock protein 105 kDa 4.88±0.73	0±0
P05783 K1C18 Keratin, type I cytoskektal 18 7.87±1.07	0±0
P04632 CPNS1 Calpain small subunit 1 6.43±0.96	0.03±0.01
P29317 EPHA2 Ephrin type-A receptor 2 6.39±2.2	0±0
P05787 K2C8 Keratin, type II cytoskeletal 8 11.78±1.3	0±0
060443 DFNA5 Non-syndromic hearing impairment protein 5 11.92±1.74	3 0.03±0.01
P02675 FIBB Fibrinogen beta chain 0.32±0.07	0±0
P09601 HMOX1 Heme oxygenase 1 0.12±0.01	0±0
000571 DDX3X ATP-dependent RNA helicase DDX3X 0.32±0.07	0.02±0.02
Q9Y3A2 UTP11 Probable U3 small nucleolar RNA-associated protein 11 0.14±0.03	
Q13573 SNW1 SNW domain-containing protein 1 0.12±0.05	
076021 RL1D1 Ribosomal L1 domain-containing protein 1 0.12±0.11	
Q6P1J9 CDC73 Parafibromin 0.27±0.03	
Q14344 GNA13 Guanine nucleotide-binding protein subunit alpha-13 0.39±0.12	
Q86SJ2 AMGO2 Amphoterin-induced protein 2 0.25±0.08	
Q8IX12 CCAR1 Cell division cycle and apoptosis regulator protein 1 0.4±0.08	0±0
Q9NZM5 GSCR2 Glioma tumor suppressor candidate region gene 2 protein 0.22±0.18	
043290 SNUT1 U4/U6.U5 tri-snRNP-associated protein 1 0.28±0.11	
Q13616 CUL1 Cullin-1 0.33±0.04	
Q9NY61 AATF Protein AATF 0.32±0.12	
P11717 MPRI Cation-independent mannose-6-phosphate receptor 0.43±0.03	
P15924 DESP Desmoplakin 0.36±0.16	0.01±0

most significantly enriched pathways. In the network, the proteins indicated with red circle nodes were up-regulated, and the proteins indicated with green circle nodes were downregulated. These data clearly show that most of the proteins were down-regulated in Ribosome biogenesis in eukaryotes, Spliceosome, and mRNA surveillance pathways, while the dysregulated proteins in Cysteine and methionine metabolism, Propanoate metabolism and Glycolysis/Gluconeogenesis were up-regulated.

Evaluation of iTRAQ results using western blotting

Based on the results of the MS analysis, the expression levels of five dysregulated proteins were validated using western blotting in TP-treated or negative control cells. The expression levels of two proteins (MTA2 and EIF4A3) were significantly down-regulated (Fig. 8A), while the expression levels of the remaining three proteins (PHB, CDH1 and AIFM1) were markedly increased in TP-treated cells compared with the corresponding control (Fig. 8B), consistent with the results from the MS analysis. Therefore, the altered expression levels of 312 proteins were considered induced by TP.

Discussion

TP has been widely investigated for its broad-spectrum anticancer activity. Many studies have shown that TP inhibits cell growth and induces apoptosis in various cancers,



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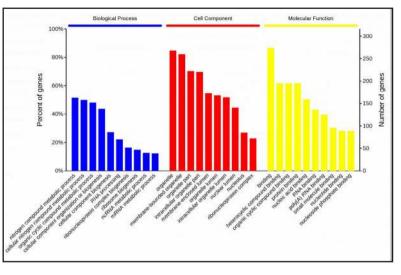
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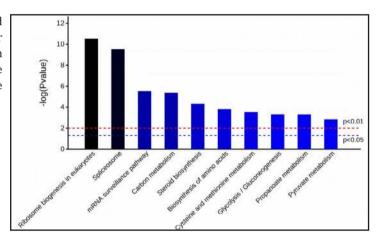
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Fig. 5. GO enrichment analysis. An overview of the GO annotations of the 312 dysregulated proteins with up to 10 significantly enriched terms in three categories: biological process (BP), cellular component (CC) and molecular function (MF). The cutoff of P-value was set to 0.05. Terms in the same category were ordered based on the P-values. Information for the percentages and numbers of involved genes/proteins in a term is provided on the left and right y-axes.

Fig. 6. Distribution of enriched KEGG pathways. Columns refer to related pathways, colored with gradient colors from midnight blue (smaller P-value) to lighter blue (larger P-value).





primarily through multiple mechanisms, including the suppression of various signaling pathways and proliferative and antiapoptotic factors in a given cell type and under specific conditions. TP has been reported to strongly inhibit the transcription of numerous proinflammatory mediators [25] and was also implicated as a potent inhibitor of NF-kappa B and a promoter of transcriptional arrest [26-30]. Recent studies have shown that TP inhibits RNA polymerase-mediated transcription by targeting transcription factors, leading to the down-regulation of certain mRNA molecules [31-33]. However, to our knowledge, there are no studies reporting the anti-proliferative and pro-apoptotic effects of TP against NSCLC cells at the proteomics level. In the present study, we attempted to investigate the potential protein targets of TP in a human NSCLC A549 lung adenocarcinoma cell line in vitro. First, we investigated the cytotoxicity of TP on A549 cells. TP strongly inhibited cell proliferation and induced cell apoptosis and cell cycle arrest in dose-dependent manners. Second, an iTRAQbased proteomics method was employed to analyze the molecular targets of A549 cancer cells after TP treatment, and pathway and network analyses were performed. Proteomics analysis is a powerful tool for the identification of biological markers and estimation of biological networks [34]. A global view of the inter-connectivity of signaling proteins and their actions is critically important for successful lung cancer therapy [35] and should provide a comprehensive perspective for elucidating the roles of TP as a potential agent for treatment of NSCLC. We observed 312 differentially expressed proteins in A549 cells after TP treatment. Moreover, bioinformatics analysis revealed that these proteins were involved in many BPs, including ribosome biogenesis, RNA processing, ribonucleoprotein



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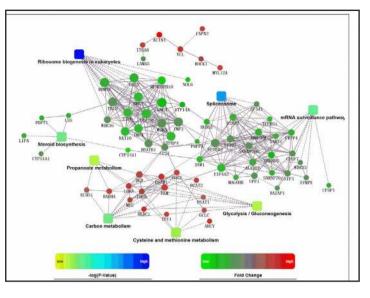
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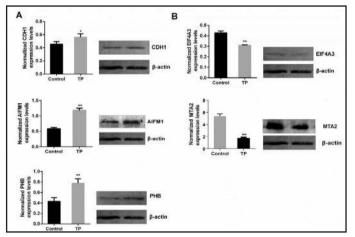
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Fig. 7. Protein-protein interaction (PPI) network. The PPI analysis was based on fold changes of protein expression, PPIs, and KEGG pathway and biological process enrichments. Circle nodes refer to genes/proteins. The rectangles refer to KEGG pathways or biological processes, colored with gradient colors from yellow (smaller P-value) to blue (larger P-value). Genes/proteins are colored in red (up-regulation) and green (down-regulation). A default confidence cutoff of 400 was used: interactions with higher confidence scores are shown as solid lines between genes/proteins or are otherwise indicated as dashed lines.

Fig. 8. Expression levels of the representative dysregulated proteins were verified using western blot analysis. (A) CDH1, AIFM1 and PHB are down-regulated proteins. (B) EIF4A3 and MTA2 are up-regulated proteins. β -actin was used as the loading control. Data are expressed as the means ± SD (n=3). *P<0.05 and **P<0.01 compared with the control group.





complex biogenesis, rRNA metabolic process, rRNA processing, ncRNA processing, cellular component biogenesis, and others. These proteins were implicated in 226 different KEGG pathways and associated with each other to form a network. The anticancer activity of TP against A549 cancer cells is mediated through its effects on multiple BPs and pathways, including ribosome biogenesis in eukaryotes, the spliceosome, mRNA surveillance pathway, PARP1/AIF pathway, metabolic pathway and other important molecular targets.

Ribosome biogenesis in eukaryotes and spliceosome and mRNA surveillance pathways are central processes for gene expression and protein synthesis, which are inextricably associated with cell growth and division. After TP treatment, most of the differentially expressed proteins involved in RNA metabolism pathways were significantly down-regulated. Among these down-regulated proteins, SNW1, HNRPM, EFTUD2, and SNRNP200 are components of the spliceosome. Many drugs have recently been demonstrated as inhibitors of RNA splicing, with cytotoxic effects on tumor cell lines [36]. SNW1 depletion induced apoptosis in breast cancer cells, and EFTUD2 knockdown also significantly promoted cellular apoptosis [37]. Previous studies have demonstrated that through the inhibition of the Akt signaling pathway, TP induces Bax- and Bcl-2-mediated mitochondrial apoptotic pathways, resulting in caspase-9- and caspase-3-triggered cell apoptosis [17, 38, 39]. In the present study, we identified key nuclear proteins and other mitochondrial proteins as down- or upstream targets of the Akt signaling pathway, such as REF1/Aly, GTPBP4, EIF4A3 and PHB.



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REF1/Aly, GTPBP4 and EIF4A3 are involved in RNA metabolism-related pathways. EIF4A3 (DDX48) is a core component of the exon junction complex (EJC) and plays a critical role in multiple posttranscriptional events, including RNA subcellular localization, nonsensemediated decay (NMD), and translation [40]. The AKT signaling pathway regulates the assembly of the core EIC proteins eIF4A3, MAGOH, and Y14 into complexes at speckled domains, which is essential for mRNA export. AKT inhibition results in the disorder of mRNA export and gene expression [41]. REF1/Aly is a nuclear speckle protein implicated in mRNA export and is a physiological target of the nuclear Akt signaling cascade [42, 43]. The depletion of Aly markedly blocks cell cycle progression and reduces cell growth and mRNA export, and these processes are regulated by Akt phosphorylation [43, 44]. GTPBP4, located in the nucleus, is involved in the biosynthesis of the 60S subunit of the ribosome [45] and can be used as a molecular switch to control signal transduction pathways, protein synthesis and other biological processes. GTPBP4 down-regulation in colorectal cancer cell lines significantly inhibited cell proliferation [46]. GTPBP4 is closely associated with the MAPK signaling pathway and participates in the regulation of MAPK and Akt signaling pathways through interactions with AKT [47, 48]. It has also been reported that GTPBP4 binds to P53, and low GTPBP4 expression leads to the aggregation and activation of P53 proteins, which regulate the downstream apoptosis-related factors caspase-3, caspase-9, and PARP [49]. Prohibitin belongs to the Band-7 protein family and is widely present in different cellular compartments. Several studies using different organism models have provided strong evidence for critical biological roles of PHB in mitochondrial function, cell proliferation, and development. The contribution of PHB to cancer cell apoptosis may depend on the stimuli and cell type [50]. Recent studies have characterized PHB as a multifunctional protein involved in the PI3K/Akt and Ras/MAPK/ERK signaling pathways. A recent study in cancer cells showed that Akt phosphorylates PHB at Thr258. PHB could also indirectly facilitate crosstalk between the PI3K/Akt and Ras/MAPK/ERK pathways through interactions with their signaling intermediates. The emerging roles of PHB in the PI3K/Akt and Ras/ERK pathways highlight the importance of PHB in the crosstalk between signaling pathways. In the present study, PHB was up-regulated in the TP-induced apoptosis of NSCLC cells. We speculate that PHB may play an important role in the TP-induced apoptosis of A549 cells. Targeting PHB may have inspiring prospects in the future research of TP anti-tumor activity.

As described above, we speculated that TP-induced toxicities in lung cancer cells may be associated with the inhibition of RNA metabolism-related pathways, regulated by the Akt signaling pathway via important molecular target proteins. TP inhibits global ribosome biogenesis and splicing in cancer cells, which may explain the high potency of TP in killing lung cancer. Further studies are required to determine the potential link between the target proteins induced by TP and lung cancer and to provide a new strategy for cancer therapy.

Apoptosis-inducing factor 1 (AIFM1) is a mitochondrial flavoprotein with a critical role in programmed cell death. AIFM1 is a cell death executioner alternative to caspases [51]. Distinguishing from classic caspase-dependent apoptosis, the PARP1/AIF death cascade is a highly orchestrated and caspase-independent programmed cell death process termed parthanatos [52, 53]. In the present study, both AIF and PARP1 were up-regulated in NSCLC A549 cells after TP treatment, indicating that in addition to the classic caspase-dependent apoptosis pathway [17], the PARP1/AIF pathway may be another mechanism for TP to induce lung cancer cell apoptosis.

Unlike normal human cells, cancer cells display metabolic reprogramming to meet cell growth and proliferation needs [54]. Altered metabolic pathways in cancer cells may be attractive targets for anticancer therapy [55]. In the present study, some proteins related to metabolic pathways were dysregulated after TP treatment. The dysregulated proteins ECHS1, HADHA, PKM, AHCY and GOT2 are located in the mitochondria, and these enzymes are involved in apoptosis initiation and development. The mitochondrion is an important organelle with multiple functions, including ATP production, lipid metabolism, developmental processes and apoptosis regulation [56]. The expression levels of these proteins were enhanced during TP-induced apoptosis, suggesting that apoptosis is an active



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and energy-consuming procedure. These results demonstrated that energy metabolism is important in TP-induced apoptosis. Further research is required to determine the potential link between the altered mitochondrial enzymes induced by TP and NSCLC and to provide a new strategy for NSCLC therapy.

Metastasis is the ultimate cause of death for most cancer patients. TP has been reported as an inhibitor of lung cancer cell migration and metastasis, but its mechanism is not clearly defined [12, 57, 58]. We observed some other differentially expressed proteins, such as MTA2 and E-cadherin (CDH1), implicated in cancer cell migration. MTA2 is a member of the metastasis tumor-associated family of transcriptional regulators and acts as a central regulator of key gene expression pathways central to metastatic dissemination [59]. MTA2 knockdown in human cancer cells significantly inhibited migration and invasion [60]. E-cadherin is the core protein of the epithelial adherens junction. The loss of E-cadherin expression is a crucial step in the epithelial-mesenchymal transition (EMT) and is involved in cancer invasion and metastasis [61]. In human tumors, E-cadherin down-regulation is frequently associated with poor prognosis [62, 63]. Interestingly, MTA2 promotes NSCLC metastasis through E-cadherin inhibition [64]. In the present study, we observed that TP not only up-regulates E-cadherin but also down-regulates MTA2, which might be a new target of the TP-mediated inhibition of A549 cell migration.

In conclusion, TP showed significant cytotoxicity in human A549 lung cancer cells, induced cell apoptosis and blocked cell cycle arrest. Potential cytotoxicity mechanisms were explored using an iTRAO-based proteomics approach. The results provided the first evidence that the broad-spectrum anti-tumor activity of TP in lung cancer cells may be associated with inhibition of RNA metabolism and protein synthesis. Among the large number of differentially expressed proteins identified, some proteins, which may be potential targets for lung cancer treatment in the future, were validated. The present study provides an effective platform for the anticancer activity of TP. However, the present study has several limitations. First, there is a lack of the same experiments on normal lung cells. Second, protein profile changes in normal lung cells in response to TP may better reveal the specificity of TP effects on cancer cells. Moreover, the *in vivo* activity, clinical application, and other mechanisms of TP against NSCLC require further investigation.

Abbreviations

TP (triptolide); PNCA-1 (pancreatic cancer cells); NSCLC (non-small cell lung cancer); iTRAQ (isobaric tags for relative and absolute quantitation); NanoLC-MS/MS (nano liquid chromatography-mass spectrometry); FBS (fetal bovine serum); DMSO (dimethylsulfoxide); BCA (bicinchoninic acid); PI (propidium iodide); PMSF (phenylmethanesulfonyl fluoride); BP (biological process); CC (cellular component); KEGG (Kyoto Encyclopedia of Genes and Genomes); MF (molecular function); SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis); PVDF (polyvinylidene fluoride); GO (Gene Ontology); HP-RP (high-pH reverse phase); CE (collision energy); FDR (false discovery rate.);

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Disclosure Statement

No conflicts of interest exist in the submission of this manuscript, and this manuscript was approved by all authors for publication.



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