Original Article
Proteomic analysis identifies proteins associated with curcumin-enhancing efficacy of irinotecan-induced apoptosis of colorectal cancer LOVO cell

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Abstract: Objective: We wish to implement a proteomics-based approach to pick and identify the proteins associated with curcumin enhancing efficacy of irinotecan inducing apoptosis of colorectal cancer LOVO cells, and further explore their synergy mechanism by bioinformatics. Methods: A colorectal cancer cell line (LOVO cell) treated by curcumin combined with irinotecan in different ways respectively was used as our comparative model. Protein spots were analyzed through MALDI-TOF/TOF. The location and function of differential protein spots were analyzed through UniProt database. Protein-protein interactions were examined through String software. Results: A total of 54 protein spots differentially expressed with 1.5-fold difference were picked, 11 of which were repeated. They mainly were involved in intracellular calcium pathways, cellular respiratory chain pathway and intracellular redox reaction pathways of LOVO cell. According to the function of various protein points, combining with varying curves of protein points in each treatment groups, we selected five interesting protein spots, 4 of which exists Protein-protein interactions, and they were close to the formation and reduction of disulfides in intracellular endoplasmic reticulum (ER). Conclusion: We selected preliminary but comprehensive data about differential expression protein spots of LOVO cell. Among these, the five interesting differential expression protein spots identified in this study may provide new insight into LOVO cell therapeutic biomarkers. Curcumin may suppress GSTM5 expression to enhance the lethal effect of irinotecan on LOVO cells, and maybe their combination via the affection of PDI and PRDX4 to disturb the formation and reduction of disulfides results in inducing apoptosis of LOVO cell.

Keywords: Curcumin, irinotecan, proteomics, colorectal cancer

Introduction

In recent years, the incidence of CRC has markedly increased in China, particularly in urban populations. And the statistics in recent years show that, as the people's living standards improving, diet changed, the incidence of colorectal cancer in China is increasing year by year [1, 2]. Chemotherapy is an important part of comprehensive treatment for patients with advanced colorectal cancer. From the late 1980s, chemotherapeutic regimen of fluorouracil in combination with leucovorin (FU/LV) became the standard first-line therapy for advanced colorectal cancer, but its response rate is only 25% [3-5], and the median survival is < 1 year [6, 7]. Since irinotecan and oxaliplatin were introduced to advanced colorectal can-

cer treatment, incorporated in to FU/LV-based regimens, FOLFIRI and FOLFOX markedly improve response rate and prolong median survival over fluorouracil with leucovorin (FU/LV) [8-10], and have supplanted FU/LV as the standard systemic approach for advanced colorectal cancer.

Irinotecan (CPT-11) is semi-synthetic camptothecin derivative extracted from chinese unique plant Camptotheca acuminata, its antitumor mechanisms is that irinotecan (predominantly in the form of SN38) binds to the Top I-DNA complex, stabilizing it and preventing re-ligation, colliding with advancing replication forks results in the formation of double stranded DNA breaks. These breaks can activate cell cycle arrest in G2 phase, if unrepaired, it can cause

cell death. The toxicity of irinotecan is especially stronger for the S phase of the cell than other phases [11]. With widely used in clinical application, it has found that the efficacy of irinotecan for the tumor cells was 49%, mainly due to their multidrug resistance [12].

Curcumin, the main chemical ingredient of turmeric, is polyphenolic compound, and its pharmacological effect includes anti-tumor, anti-inflammatory, anti-oxidation, anti-atherosclerotic, anti-HIV [13-15]. Previous studies have found that curcumin could enhance the efficacy of the chemotherapy drugs through different mechanisms. It has found that curcumin can inhibit the expression of the epidermal growth factor receptor (EGFR) and insulin-like growth factor 1 receptor (IGF-1R), resulted in enhancing killing effect of 5-FU and oxaliplatin to colon cancer HCT-116 and HT-29 cell lines [16]. It was also found that curcumin could enhance the efficacy of irinotecan inhibiting the growth of LOVO cells [17].

In this study, we planned to implement a proteomics-based approach to pick and identify the proteins associated with curcumin enhancing efficacy of irinotecan inducing apoptosis of colorectal cancer LOVO cell, and further explore their synergy mechanism by bioinformatics.

Materials and methods

Cell culture

Colorectal cancer LOVO cells (ATCC, USA) were used in this study. LOVO cells were cultured as monolayers in RPMI 1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, Brazil) and 1% penicillin/streptomycin. Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2.

Experimental groups and drug intervention

Experimental groups: Divided into five groups, each set up three parallel groups, grouped as follows:

Group 1: Control group (no drug intervention group).

Group 2: Irinotecan monotherapy group (treated by irinotecan for 48 h).

Group 3: Curcumin monotherapy group (treated by curcumin for 48 h).

Group 4: Mixed-administered group (treated by curcumin mixed with irinotecan together for 48 h).

Group 5: Sequential treatment group (first treated by curcumin for 48 h, then transformed into irinotecan for 48 h).

Drug intervention: LOVO cells at 5×10^6/dish were expanded to 15 cm cell culture dishes until the cells reached 90% confluence, then washed with sterile PBS for drug intervention. In our Preliminary works, we have been selected the half-inhibitory concentration (IC_{50}) of curcumin and irinotecan on LOVO cells by the MTT assays for 48 h, which the half-inhibitory concentration of curcumin on LOVO cells for 48 h is 10.3 µg/ml, and of irinotecan is 21.2 µg/ml. Drug intervention according to the methods above.

Sample preparation for DIGE

Cells were first harvested, washed with PBS, and pelleted by centrifugation and stored at -80°C until use. Frozen cells samples were weighed (100 mg/mL lysis buffer) and thawed on ice. And then the samples were resuspended in Lysis Buffer (30 mM Tris-HCl, 7 M urea, 2 M thiourea, 4% CHAPS, at pH 8.5), and incubated on ice for 30 minutes. Suspensions were sonicated on ice during sonication to prevent sample heating and used 10 seconds bursts with 30 seconds pauses for 5 times. Lysates were then centrifuged at 12,000 g for 30 minutes. The suspension proteins were then precipitation with 2D Clean-up Kit (GE Healthcare) according to the manufacturer's protocol and resuspended in Lysis Buffer. Concentration was determined by 2D Quant Kit (GE Healthcare) according to the instruction. Protein was aliquoted to required amounts, frozen or freeze dried. All reagents were supplied by Sigma Chemical Company unless otherwise noted.

Differential in-gel electrophoresis (DIGE)

For DIGE, 50 µg of proteins were minimally labelled with CyDyes at the ratio of 50 µg proteins: 400 pmol Cy3 or Cy5 protein-labeling dye (GE Healthcare) according to the manufacturer's protocol. Cy3 and Cy5 were used for samples, Cy2 was used for the internal standard (a pool of all samples). Each labeled sample was mixed with rehydration buffer (7 M urea, 2 M
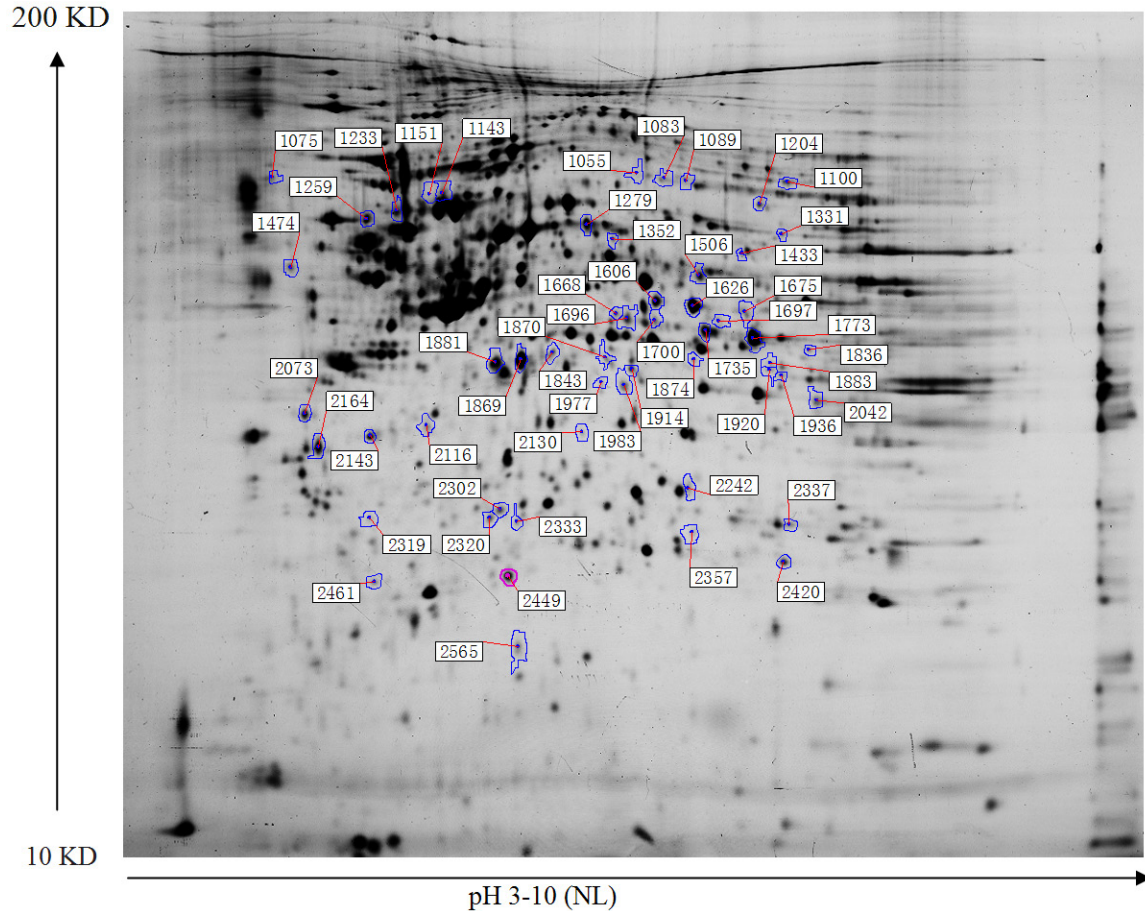
Figure 1. Location of 54 picked protein spots in the 2-D gel.

thiourea, 4% CHAPS, 2% dithiothreitol, 2% Pharmalyte; GE Healthcare) and applied to a 24 cm immobilized pH gradient gel strip (immobilized pH gradient (IPG) strip pH 3 to 10 NL) for separation in the first dimension. First dimension isoelectric focusing was carried out at 20°C in IPGphor III (GE Healthcare) using the following protocol: 30 V for 12 hours; 1,000 V gradient for 2 hours; 8,000 V gradient for 2 hours; then at 8,000 V for 60,00 volt-hours followed by a 500 V hold. After isoelectric focusing, strips were equilibrated by agitating for 15 minutes in 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS), 650 mM DTT and then agitating for 15 minutes in 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 1.27 M iodoacetamide. The strips were next loaded onto a 24 × 24 cm 12% polyacrylamide gel using low fluorescence glass plates and subjected to an electric field in DALT Six (GE Healthcare; 15°C at a constant 3 W per gel, 12

hours in a running buffer containing 25 mM Tris, 192 mM glycine, and 0.1% (w/v) SDS). After the 2-DE run, gels were scanned on a Typhoon 9400 imager (GE Healthcare) and analyzed with DeCyder 2D Software V6.5 (GE Healthcare). Protein spots, which were found differentially expressed with 1.5-fold difference between groups were interesting spots. Samples for the spot picking gel were prepared without labeling by Cydyes, a preparative gel was run using 600 μg of pooled protein sample and stained with colloidal Coomassie blue G-250. Matched spots of interest were picked automatically from the preparative gel by Ettan Spot Picker (GE Healthcare).

Protein identification

The picked spots were destained with 50% acetonitrile (ACN)/100 mM NH_4HCO_3 for 10 min, dehydrated with 100% ACN for 10 min, and then dried using a centrifugal concentrator

Proteomic analysis for colorectal cancer LOVO cell

Table 1. Proteins with CV values were identified by MALDI-TOF/TOF

Spot score no.a)	Protein name	Access. no.b)	Theoretical		Protein score	Protein (C.V%)
			Mass (Da)	pI		
1055	Cytokeratin-9	P35527	62254.9	5.14	46	52.33
*1075	Calnexin	P27824	67982	4.47	75	99.941
1083	Cytokeratin-9	P35527	62254.9	5.14	50	79.665
1089	Keratin, type I cytoskeletal 10	P13645	59045.8	5.09	140	100
Φ1100	Flavoprotein subunit of complex II	P31040	73671.7	7.06	268	100
1143	Gamma-actin	P63261	42107.9	5.31	636	100
1151	Gamma-actin	P63261	42107.9	6.57	226	100
1204	T-complex protein 1 subunit zeta	P40227	58443.8	6.23	53	88.825
*1233	Nucleobindin-1	Q02818	53846.3	5.15	173	100
&1259	Protein disulfide-isomerase	P07237	57479.8	4.76	59	99.975
1279	Cytokeratin-8	P05787	53671.1	5.52	98	100
1331	Keratin, type I cytoskeletal 10	P13645	59045.8	5.09	122	100
1352	Heterogeneous nuclear ribonucleoprotein H	P31943	49483.5	5.89	278	100
1433	TBP-interacting protein	Q9Y265	50538.4	6.02	212	100
1474	Cytokeratin-9	P35527	62254.9	5.14	41	0
1506	26S proteasome non-ATPase regulatory subunit 11	O00231	47719.1	6.08	38	0
1606	Serpin B3	P29508	44593.6	6.35	27	0
1626	Choline-phosphate cytidylyltransferase A	P49585	42047	6.82	37	0
1668	Actin regulatory protein CAP-G	P40121	38778.6	5.88	97	100
1675	L-2-hydroxyglutarate dehydrogenase, mitochondrial	Q9H9P8	51068.2	8.57	40	0
1696	S-arrestin	P10523	45262.5	6.14	33	0
1697	Septin-2	Q15019	41689.3	6.15	138	100
1700	S-arrestin	P10523	45262.5	6.14	38	0
1735	Heat shock transcription factor, Y-linked	Q96LI6	45249.7	6.68	43	7.051
1773	Heterogeneous nuclear ribonucleoprotein M	P52272	77749.4	8.84	29	0
1836	Keratin, type II cytoskeletal 1	P04264	66170.1	8.15	43	0
1843	Keratin, type I cytoskeletal 9	P35527	62254.9	5.14	72	99.859
1869	E3 ubiquitin-protein ligase	Q969Q1	41134	4.85	34	0
1870	Keratin, type I cytoskeletal 16	P08779	51578.3	4.99	94	100
1874	Heat shock transcription factor, Y-linked	Q96LI6	45249.7	6.68	33	0
1881	F-actin-capping protein subunit alpha-1	P52907	33073.4	5.45	140	100
*1883	Annexin A1	P04083	38918.1	6.57	158	100
Φ1914	Ubiquinone biosynthesis methyltransferase COQ5	Q5HYK3	37401.9	6.47	33	0
*1920	Annexin A1	P04083	38918.1	6.57	138	100
1936	Heterogeneous nuclear ribonucleoprotein H3	P31942	36960.1	6.37	83	99.991
1977	Protein phosphatase 1K, mitochondrial	Q8N3J5	41484.8	6.27	25	0
1983	Inorganic pyrophosphatase 2, mitochondrial	Q9H2U2	38409.2	7.07	43	4.886
2042	Zinc finger protein 550	Q7Z398	49490.5	8.95	33	0
2073	Keratin, type II cytoskeletal 1	P04264	66170.1	8.15	116	100
&2116	Glutathione S-transferase Mu 5	P46439	25829.3	6.9	35	0
2130	Keratin, type I cytoskeletal 9	P35527	62254.9	5.14	48	67.771
*2143	Annexin A5	P08758	35971.4	4.94	255	100
2164	Keratin, type I cytoskeletal 10	P13645	59045.8	5.09	108	100
2242	Septin-14	Q6ZU15	50449.4	5.87	31	0
&2302	Peroxiredoxin-4	Q13162	30748.9	5.86	321	100
*2319	Calpain small subunit 1	P04632	28468.8	5.05	55	93.999
2320	Diphthine--ammonia ligase	Q7L8W6	30688.5	5.34	25	0
Φ2333	NADH-ubiquinone oxidoreductase 30 kDa subunit	O75489	30336.8	6.99	105	100
2337	Sulfotransferase 1C4	O75897	35667.9	8.22	50	81.022

Int J Clin Exp Pathol 2014;7(1):1-15

&2357	Glutathione S-transferase Mu 5	P46439	25829.3	6.9	30	0
2420	Sulfotransferase 1C4	O75897	35667.9	8.22	31	0
&2449	Peroxiredoxin-2	P32119	22049.3	5.66	329	100
Ф2461	NADH-ubiquinone oxidoreductase 23 kDa subunit	O00217	24202.8	6.00	90	99.998
*2565	Translocon-associated protein subunit delta	P51571	19157.7	5.76	109	100

This table is based on **Figure 1**, different protein spots are separated by 2-D electrophoresis in the combination of curcumin and irinotecan in different manners on the effects of LOVO cells, and then use MALDI-TOF/TOF to identify various spots. Among them, points marked *refers to proteins associated with calcium ion channel pathway; spots marked &refers to proteins associated with the cellular respiratory chain pathway; spots marked Фrefers to proteins associated cellular redox reaction pathway; Spots marked Red are interesting protein spots selected in our study, intends to further explore the function that these five spots play in synergy mechanism curcumin combined with irinotecan.

(TOMY SEIKO, Tokyo). Next, 2 µl of 25 ng/ml trypsin (Promega) diluted in 50 mM NH_4HCO_3 was added to each gel piece and incubated for 30 min at 4°C, and then 30 µl of 50 mM NH_4HCO_3 was added, and then the spots were incubated overnight at 37°C. We used two solutions to extract the resulting peptide mixtures from the gel pieces. First, 100 µl of 60% (v/v) ACN in 0.1% aqueous trifluoroacetic acid (TFA) was added to the gel pieces, which were then sonicated for 15 min. Next, we collected the solution and added 50 µl 100% ACN for the last extraction. Digested peptides were dried with Vacuum Pump and dissolved using 2 µl 50% acetonitrile/0.1% TFA, and aliquots of 0.5 µl were applied on the target disk and air-dried. Then 0.5 µL of matrix solution (CHCA saturated in 50% acetonitrile/0.1% TFA) was added to the dried samples and again allowed to dry. Samples on the MALDI target plates were then analyzed by ABI 4800 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems). For MS analyses, typically 800 shots were accumulated. MS/MS analyses were performed using air, at collision energy of 2 KV. MASCOT search engine (version 2.1, Matrix Science) was used to search all of the tandem mass spectra. GPS Explorer™ software version 3.6.2 (Applied Biosystems) was used to create and search files with the MASCOT search for peptide and protein identification. Protein identities were obtained by using Mascot searching engine against Swiss-Prot non-redundant sequence databases selected for human taxonomy.

Bioinformatics

The theoretical isoelectric point (pI) and molecular weight (MW) of protein spots were calculated through MALDI-TOF/TOF. The sub-cellular location and function of the identified proteins were elucidated by UniProt knowledgebase (Swiss-prot/TrEMBEL). A protein-protein interactions network was done by STRING software9.05 through inputting IPI number http://string.embl.de.

Results

The main purpose of this study is to define synergy mechanism that curcumin combined with irinotecan pass though different approach effect on colorectal cancer LOVO cells in vitro, by using 2-D electrophoresis to filter out the differential expression of protein spots between the groups, and then identified protein function by MALDI-TOF/TOF.

In our study, we adopted 2-D electrophoretic separation of proteome of LOVO cell in different groups, and then by DeCyder 2D Software V6.5, according to the screening principle that differential expression decreasing or increasing 1.5-fold, after the comparison between the different groups, we picked a total of 54 differentially expressed proteins, the position of each spot was shown in **Figure 1**, on this basis, we further identified all these 54 protein spots by MALDI-TOF/TOF, and found that 11 protein spots were repeating. Information about each spot was shown in **Table 1**. According to function of all these 43 protein spots, we found that the combination of curcumin and CPT-11 mainly affect intracellular calcium pathways, cellular respiratory chain pathway and intracellular redox reaction pathways of LOVO cell. Among them, proteins associated with intracellular calcium ion channel pathway include Calnexin, Nucleobindin-1, Annexin A1, Annexin A5, Calpain small subunit 1, Translocon-associated protein subunit delta (protein spots marked * shown in **Figure 1**); Related to intracellular respiratory chain pathway contain NADH-ubiquinone oxidoreductase 30 kDa subunit, NADH-ubiquinone oxidoreductase 23 kDa subunit, Ubiquinone
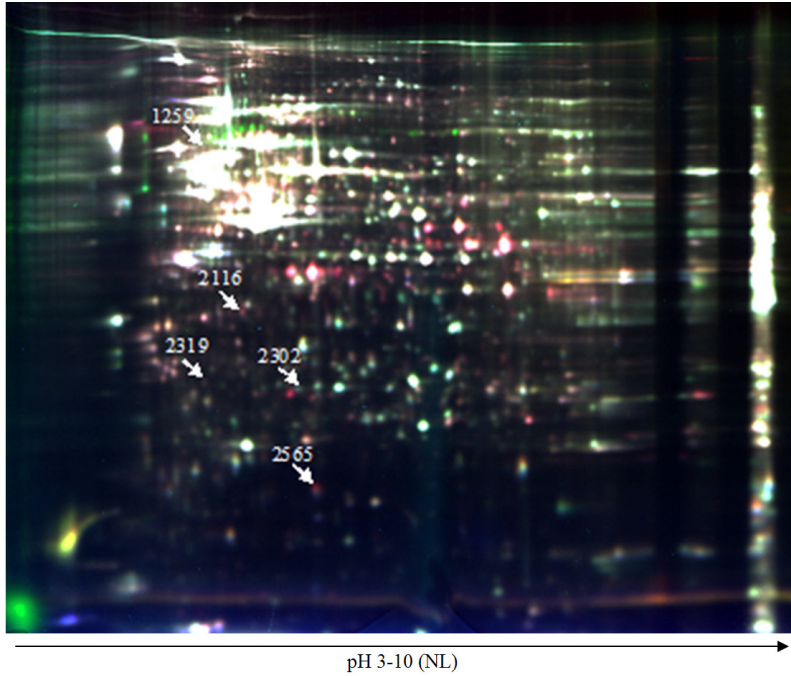
pH 3-10 (NL)

Figure 2. The position of five interesting protein spots in 2-DIGE image. A representative 2-D DIGE image of samples from the five groups (24 cm, pH 3-10, NL). Cy2 (blue) image of proteins from an internal standard is the pool of all the samples, Cy3 (green) image of proteins from NC group, CPT-11 monotherapy group, sequential treatment group, and Cy5 (red) image of proteins from curcumin monotherapy group, mixed-administered group. Spot numbers correspond to those in **Table 1**.
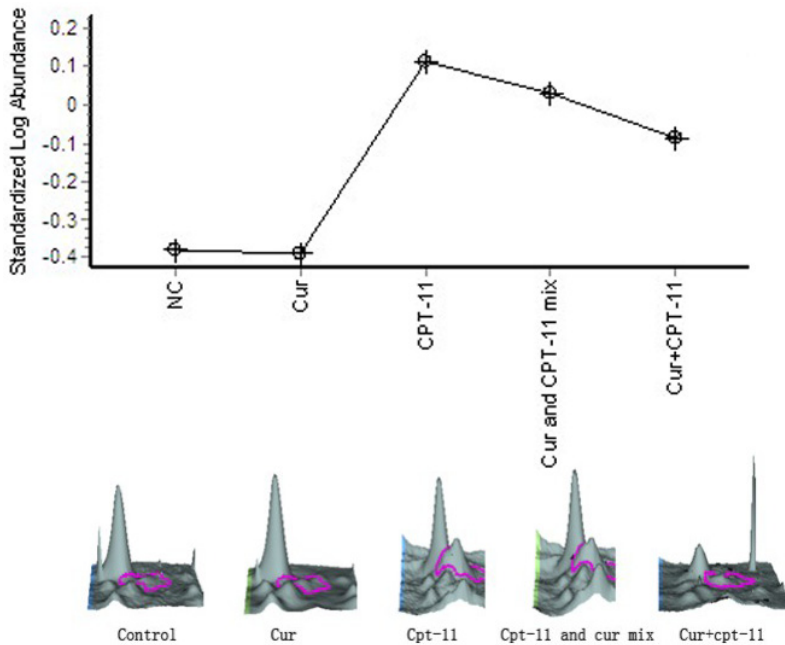

Figure 3. Glutathione S-transferase Mu 5.

biosynthesis methyltransferase COQ5, Flavoprotein subunit of complex II (spots marked &

shown in **Figure 1**), and correlated to Intracellular redox reaction pathway cover Peroxiredoxin-4, Peroxiredoxin-2, Glutathione S-transferase Mu 5, Protein disulfide-isomerase (spots marked Φ shown in **Figure 1**), Searching by Uniprot Database, we initially understood the function of each protein spot, combined with our experimental purposes and protein expression changing curve, we picked five interesting protein spots, which included Glutathione S-transferase Mu 5 (GSTM5), Peroxiredoxin-4 (PRDX4), Calpain small subunit 1 (CAPNS1), Translocon-associated protein subunit delta (SSR4) and Protein disulfide-isomerase (P4HB), the positions of the five proteins in 2-D DIGE image were shown in **Figure 2**.

For the changing curve of Glutathione S-transferase Mu 5 expression between different groups (**Figure 3**), we found that, compared with the negative control group, after curcumin treatment, $GSTM_5$ expression was downregulated 1.03-fold, however, after be treated by irinotecan, $GSTM_5$ expression was upregulated 3.10-fold, compared with irinotecan monotherapy group, $GSTM_5$ expression in the mixed-administered group and sequential group was gradually decreased by 1.21-fold, 1.58-fold respectively.

According to Peroxiredoxin-4 (PRDX4) variation curve (**Figure 4**), we found that, compared with the negative control group, PRDX4 expression was upregulated in curcumin monotherapy group,
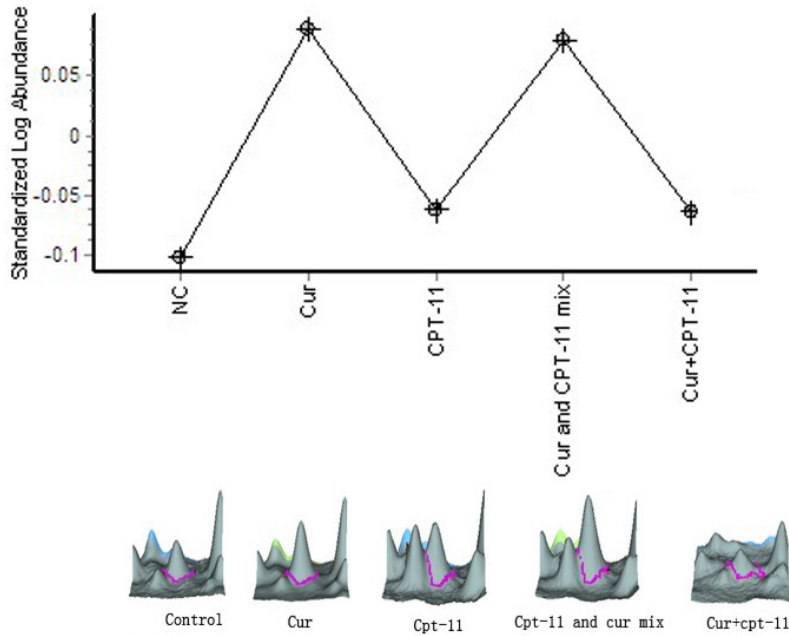
Figure 4. Peroxiredoxin-4.
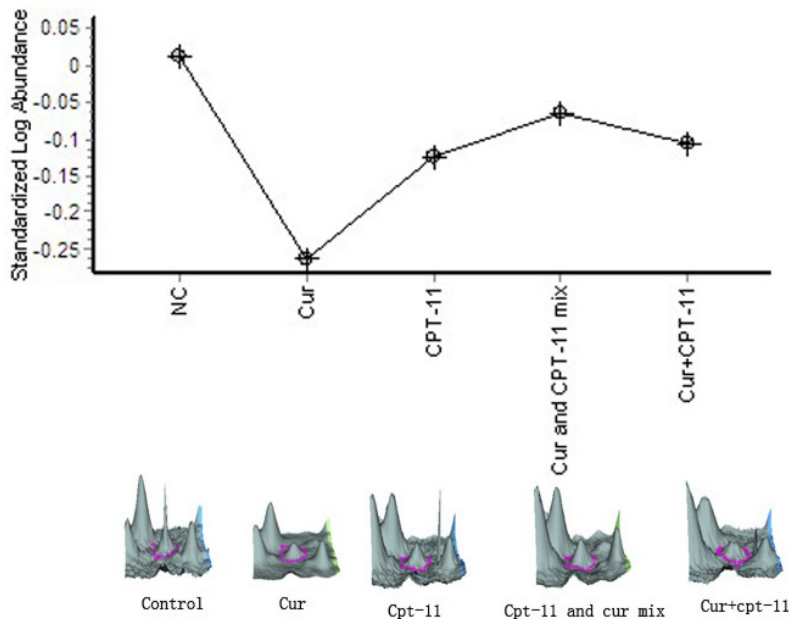
Figure 5. Calpain small subunit 1.

apy group, PRDX4 expression in the mixed-administered group increased in multiples of 1.38, but which in the sequential administration group decreased by 1.0-fold.

Base on Calpain small subunit 1 (CAPNS1) variation curve (**Figure 5**), we found that, compared with the negative control group, CAPNS1 expression in each treatment group was down-regulated, of which in the curcumin, irinotecan monotherapy group were reduced by 1.89-fold, 1.37-fold respectively, while in the mixed-administered and sequential group, CAPNS1 expression was gently increased by 1.38-fold, 1.04-fold respectively in contrast to irinotecan monotherapy group, shows that the effect of curcumin for inhibiting CAPNS1 expression was the most significant among all groups, however, when we used the combination of curcumin with irinotecan to treat LOVO cell in vitro, the result showed that not only they did not show their synergistic effect in suppressing CAPNS1 expression, but also display their antagonism, this implies the mechanism the two drugs play in inhibiting the expression of CAPNS1 may be not the same.

irinotecan monotherapy group, mixed-administered group and sequential administration group, of which the most significant effect was in the curcumin monotherapy group and mixed-administered group, the ratio of PRDX4 expression in the curcumin, irinotecan monotherapy groups compared with the negative control group were upregulated by 1.55-fold, 1.09-fold respectively, compared to irinotecan monother-

On the basis of Translocon-associated protein subunit delta (SSR4) variation curve (**Figure 6**), we found that, the changing curve was similar to that of GSTM5, compared with the negative control group, SSR4 expression in the curcumin monotherapy group was decreased by 1.67-fold, while in the irinotecan monotherapy group was increased by 2.44-fold. When using combination of two drugs in
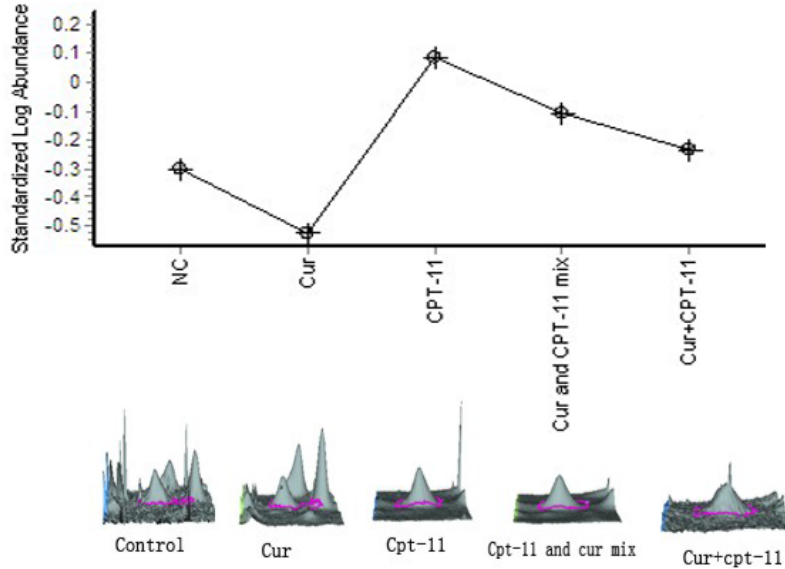
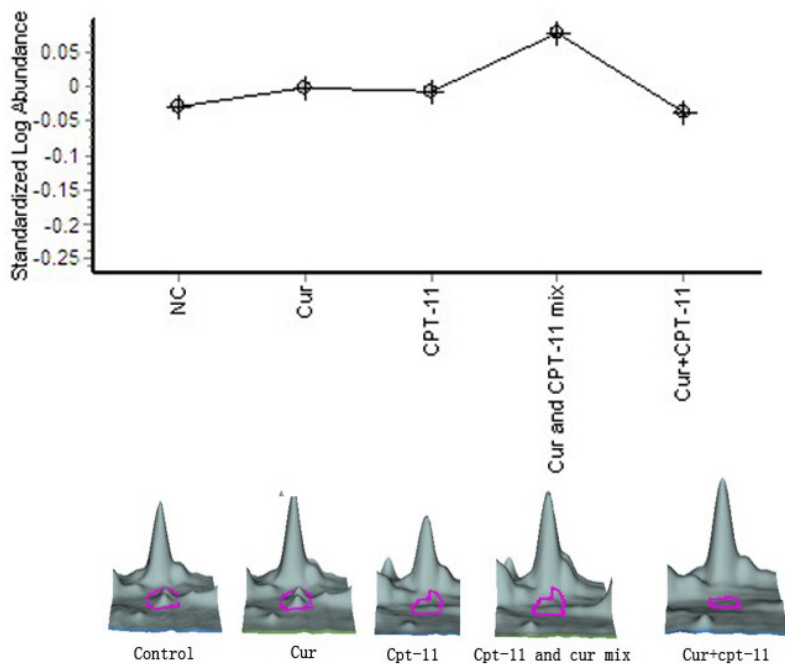Figure 6. Translocon-associated protein subunit delta.

Figure 7. Protein disulfide-isomerase. These five Pictures above are changing curves and three-dimensional maps of five interesting spots selected in this study in different groups, diagram showing the various trends under different treatments, in which the three-dimensional map marked by red arrows and pink circles are interesting proteins.

In the light of protein disulfide-isomerase (P4HB) changing curve (**Figure 7**), we found that, compared with the negative control group, P4HB expression between curcumin, irinotecan monotherapy group was no significant, and curcumin, irinotecan monotherapy gently raised P4HB expression at 1.06-fold, 1.05-fold respectively, compared to irinotecan monotherapy group, P4HB expression in mixed-administered group was increased by 1.22-fold, while in the sequential group was slightly reduced at 1.07-fold.

Based on database STRING, we use STRING9.05 software to search for interactions between the 43 protein spots, which were shown in **Figure 8**, the score of the required confidence according to STRING9.05 software searching for protein-protein interactions: Low confidence: 0.150; medium confidence: 0.400; high confidence: 0.700; highest confidence: 0.900. In this study, we found that, the score of interaction between P4HB with PRDX4 is 0.682, between SSR4 with PRDX4 the score is 0.602, the score for interaction between P4HB with CAPNS1 is 0.418, in accordance with its standard division, the score for interaction between these four proteins is between medium confidence and high confidence, which illustrates that these four protein spots have a close relationship, and involving intracellular signaling pathways is still worth our further exploration.

mixed-administered and sequential administration ways, we found the SSR4 expression was gradually inhibited in this two groups, reduction at 1.57-fold, 2.08-fold, which was compared to irinotecan monotherapy group.
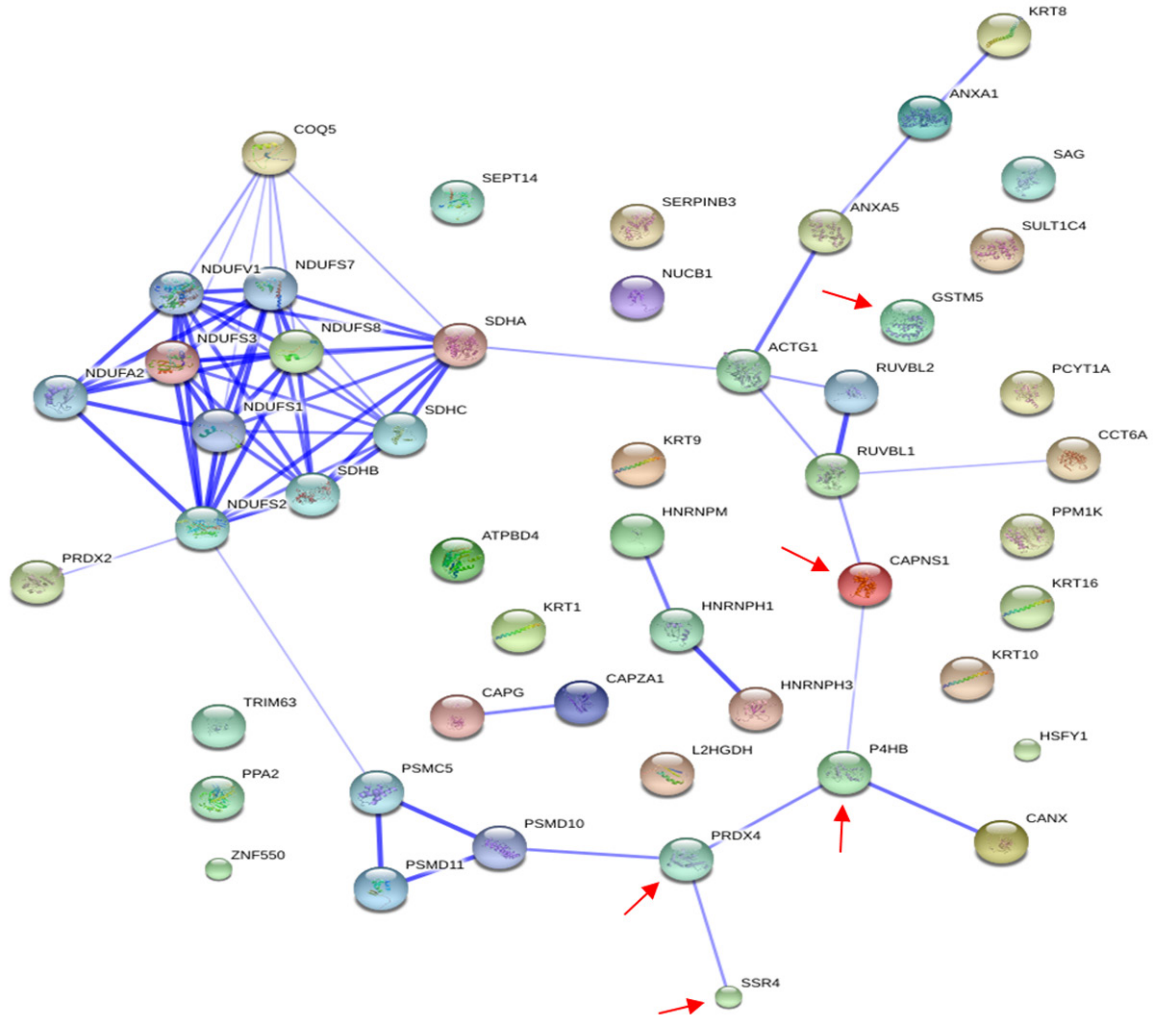
Figure 8. Interaction graph of 43 protein spots. This figure is based on the database STRING, by using software STRING software9.05 to generate automatically interaction graph of 43 protein spots, in which marked by the red arrow are interesting spots in our study.

Discussion

The purpose of this study is to find out targets of curcumin and irinotecan acting on LOVO cells, and further to pick protein spots abnormally expressed in LOVO cells treated with irinotecan and curcumin in different combinational manners. Thus we can figure out the synergy mechanism of these two drugs to LOVO cells. By 2D electrophoresis, we found that, compared with negative control group, curcumin monotherapy group, irinotecan monotherapy group, mixed-administered group and sequential treatment group could change the expression of protein points in LOVO cells. We screened out a total of 54 differential expressed protein points by mass spectrum identification,

11 of which were repeated ones. Through the retrieval of Uniprot database, we preliminarily made clear the function of each protein point According to the function of various protein points, combining with their varying curves in each treatment group, we selected five interesting protein spots, including Peroxiredoxin-4, Glutathione S-transferase Mu 5, Translocon-associated protein subunit delta, Calpain small subunit 1 and Protein disulfide-isomerase. Furthermore, by using STRING database to retrieve the relationship among these proteins above, we found that there exists protein-protein interactions among Peroxiredoxin-4, Translocon-associated protein subunit delta, Calpain small subunit 1 and Protein disulfide-isomerase. Peroxiredoxin-4, Protein disulfide-

isomerase and glutathione are involved in the oxidation-reduction reaction pathway in the endoplasmic reticulum. Peroxiredoxin-4, through the consumption of its mercapto group, generates disulfide bonds and catalyzes hydrogen peroxide into water in the endoplasmic reticulum, to maintain the steady-state of endoplasmic reticulum in cells. Protein disulfide-isomerase (PDI) family play an important role in the formation and reduction of disulfides, the native disulfides formation is very important for the protein stability [18-20]. About the five interesting protein spots selected above, we further explore the synergistic mechanisms between the two drugs to LOVO cells. The pathways PDI family members involve in the formation and reduction of disulfides was thoroughly discussed in the review "Multiple ways to make disulfides" by Bulleid NJ, Ellgaard L [18]. From this review, we got the information about the formation and reduction of disulfides in intracellular endoplasmic reticulum (ER), and also understood that Protein disulfide-isomerase (PDI) family plays an important role in the formation and reduction of disulfides, the native disulfides formation is very important for the protein stability. What interested us is that PRDX4, PDI, GSH and FADH2 which are closed to our research result all participate in the formation of disulfides. Base on this review, we believe the interaction between PDI and PRDX4 is that they all involve in the formation of disulfides, and according to our present research result, maybe the combination of curcumin and irinotecan via the affection of PDI and PRDX4 to disturb the formation of disulfides, and results in inducing apoptosis of LOVO cell, which remains to be further explored.

Glutathione S-transferase Mu 5

Glutathione S-transferase family, chiefly located in the cytoplasm, mainly includes Alpha, Mu, Pi and Theta subtribe. Among them, Mu subtribe can also be divided into M1, M2, M3, M4, M5 and M6 [21]. Glutathione transferase in cells has two primary functions: 1, removing the reactive oxygen species (ROS) in the body; 2, detoxification. In Oxidative stress environment, the expression of GST is up-regulated under regulation of nuclear factor-related factor 2 (Nrf2). GST clears ROS to protect the cell by catalyzing the combination of GSH (glutathione) and ROS. The present studies largely focus on the role of GST in multidrug resistance of

cancer cells to chemotherapeutic drugs, namely, the detoxification of GST. GST can reduce the killing effect of chemotherapeutics to tumor cell by combining with chemotherapeutic drugs and forming GSH-chemotherapeutic drugs compound, which increases the water solubility of chemotherapeutics and promotes their excretion from cells. Besides, there exists a coordinated expression between GST and ABC Family in tumor cells. When tumor cells are resistant to chemotherapeutics, the expression of these two proteins is both increased. ABC family can also reduce the concentration of chemotherapeutic drugs in tumor cells and decrease their efficacy by transporting GSH-chemotherapy drugs compound out from cancer cells. Besides, due to the effect of adjustment of the tumor cells to protect themselves, glutathione S-transferase within tumor cells can highly express in secondary, which is also an important cause of the secondary resistance of tumor cells to chemotherapeutic drugs [22, 23]. Glutathione S-transferase Mu 5, belonging to Mu subtribe, mainly plays a role of detoxification against foreign and endogenous substances in the body [24, 25]. Studies have found that the expression of Glutathione S-transferase Mu 5 in liver of cynomolgus macaque could be up-regulated after the injection of estrogen [26]. In this study, we discovered that the expression of Glutathione S-transferase Mu 5 in LOVO cells was up-regulated after treated with irinotecan. Accordingly, we consider that, after treated with irinotecan, LOVO cells start their own protection mechanisms and raise the expression of intracellular Glutathione S-transferase Mu 5, which increases the water-soluble ability of intracellular irinotecan and promote its efflux. With the concentration of irinotecan decreasing, its ability of LOVO cell killing descends. This principle is inferred on the basis of previous studies and this study. The verification of gene knockout and overexpression of Glutathione S-transferase Mu 5 in LOVO cells conduct remain undone in this research, which is the inadequacy of this research. Interestingly, we found that curcumin could reduce the expression of Glutathione S-transferase Mu 5 as well as the combined application of curcumin and irinotecan. Previous studies have found that curcumin could combine with glutathione to generate a conjugated compound, which may restrain the activity of MRP / GSH-X pump and then reduce

intracellular chemotherapeutics efflux mediated by this pump, to increase drug concentration in the cell. In addition, curcumin, through Michael addition reaction, consumes glutathione transferase in tumor cells to reduce the amount of GST [27, 28]. In the present study, we have not yet ensured that if curcumin suppressed the expression of Glutathione S-transferase Mu 5 via the same mechanism above. However, on the basis of our study and combined with our previous findings, we propose that curcumin may enhance the lethal effect of irinotecan on LOVO cells through lowering the expression of Glutathione S-transferase Mu 5.

Peroxiredoxin-4

Peroxiredoxin widely exists in various organs in the body. PRDX family has six members, including Peroxiredoxin-1, Peroxiredoxi-2, Peroxiredoxi-3, Peroxiredoxin-4, Peroxiredoxin-5 and Peroxiredoxin-6 [29]. In cells, the principal function of Peroxiredoxin is to remove reactive oxygen species (ROS), which plays the role in protecting cells. The study has found that PRDX was closely related to the occurrence and development of tumor. Because of high metabolic characteristics of tumor cells, there generally exist hypoxic microenvironments in most tumor cells. In oxidative stress environment, tumor cells produce reactive oxygen species (ROS) in their own, which is toxic to cells and can induce tumor cells apoptosis. By catalyzing the decomposition of ROS and clearing it in cells, peroxidase plays a protective role in tumor cells. Besides, PRDX has an important effect in the occurrence of tumor cell as well. One of the significant characteristics of cancel cells is their endless division and proliferation. The reason is that in the cell cycle cancer cells can escape cell cycle monitoring and do not enter normal cell apoptosis cycle. In general, through the normal division and proliferation, normal cells become senescent cells, and thus go into senescence and apoptosis pathway under relevant factors' regulation. If senescent cells fail to enter the normal way of decline and fall due to genetic mutation, then they will transform into tumor cells. In this cancerous transformation, ROS also play an important part because reactive oxygen species can spur senescent cells transform into tumor cells. While PRDX family can remove the senescent cells in body by their anti-oxidant ability, and

inhibit the transformation. And in hypoxic microenvironment of tumor cells, through oxidative stress effect, nuclear factor-related factor 2 (Nrf2) can bind with PRDX promoter, trans-activating the promoter domain and prompting transcription of PRDX, results in increasing PRDX expression. Therefore, as the oxidative stress monitor inside the cell, PRDX is very important for protecting both normal cells and tumor cells, and it may be one target for anti-cancer drugs in the future [30-32]. In this study, we found that the expression of Peroxiredoxin-4 in LOVO cells was both increased after disposed by curcumin and irinotecan, and, compared with curcumin, irinotecan more significantly raised expression of Peroxiredoxin-4. According to previous research, Peroxiredoxin-4 was in a state of high expression in colon cancer tissues, and the downward expression of Peroxiredoxin-4 can induce apoptosis of colon cancer HCT-116 cells [33], which shows that Peroxiredoxin-4 can maintain the growth of tumor cell and anti-tumor effect can be achieved with the inhibition of its expression. In this study, based on the anti-tumor effect of curcumin and irinotecan, we presumed that curcumin and irinotecan may suppress the expression of Peroxiredoxin-4 to promote LOVO cell apoptosis. But we are confused with the experimental result which displayed that the expression of Peroxiredoxin-4 was up-regulated after treated by curcumin and irinotecan in LOVO cells. Respecting that LOVO curcumin could restrain the intracellular respiratory chain pathway, we assume that curcumin may via inhibiting the aerobic respiration of LOVO cells to raise Peroxiredoxin-4 expression, and it also suggests that Peroxiredoxin-4 may not be the direct target of irinotecan and curcumin, but as a powerful weapon of LOVO cells to resist the killing effect of irinotecan and curcumin, and lethal effect of curcumin and irinotecan to LOVO cells may be enhanced when Peroxiredoxin-4 expression was suppressed.

Calpain small subunit 1

Calpain family, a kind of calcium dependent protease in cytoplasm and activated by combined with the calcium ions, regulates the stretching, migration, death, transcription, and signal transduction in cells. In mammalian cells, calpain 1 and calpain 2 are the major species of the calpain family [34]. They differ mainly in their sensitivity to calcium ion in vitro.

Calpain 1 is activated by low micromolar free calcium ions. Studies have found that it has a close relationship with tumor's occurrence and development, and mediates the invasion and metastasis of tumor cells. In lung cancer cell lines, it is found that the inhibition of the activity of calpain 1 could restrain the cell invasion and metastasis [35]. In breast cancer cells (SKBr3, BT474), prostate cancer cells (PC3) and colon cancer cells (HCT116), it is found that Capns1 had interaction relationships with RasGAP and participated in regulating the cell invasion and survival. What's more, Capns1 gene promoter is regulated by the Nuclear respiratory factor 1 (NRF-1) and Activator Protein 1 (AP-1), so the inhibition of NRF-1 and AP-1 can down-regulate the expression of Capns1 [36, 37]. In the present study, we found that curcumin and irinotecan both efficiently suppressed the expression of Capns1 in LOVO cells, especially the former. In the mixed-administered and sequential group, the expression of Capns1 is inhibited, which can be explained that irinotecan and curcumin can restrain the expression of Capns1 together. However, it was not clear that whether Capns1 played a same role in inhibiting apoptosis and promoting the invasion and metastasis of LOVO cells, just like in non-small cell lung cancer, breast cancer, and prostate cancer cells. In addition, whether curcumin and irinotecan reduce the expression of Capns1 by inhibiting NRF-1 and AP-1 or not remains our further study.

Translocon-associated protein subunit delta

Translocon-associated protein subunit delta, together with TRAP-alpha, TRAP-beta, TRAP-gamma, forms the translocon-related protein. Among them, translocon-related protein, a conventional protein located in the membrane of endoplasmic reticulum, mainly helps calcium ions get into the endoplasmic reticulum by coupled with them [38]. Currently only a few studies focus on TRAP-delta, but it has been affirmed that human TRAP-delta located at the q28 region of X chromosome, an area also encoding isocitrate dehydrogenase 3γ subunit (IDH3G), and both of them are subject to the regulation of a 133 bp, CpG-embedded bidirectional promoter, indicating that there may be exist some similarities between these two ones. IDH3G acting as the rate-limiting enzyme in the tricarboxylic acid cycle, has been proven that it can regulate the metabolism of cancer

cells, particularly hypoxia of tumor cells [39]. But whether TRAP-delta own a similar function or not has not been confirmed yet. In the UV-induced melanoma tumors, it is found that the expression of TRAP-delta is associated with tumor staging. At the advanced tumors stage, the expression of TRAP-delta is higher [40]. And in melanoma cell lines TD6b and TD15L2, TRAP-delta is also found at high expression levels. Thus it suggests that TRAP-delta may promote the occurrence and metastasis of melanoma induced by ultraviolet rays, and it also can be explained that TRAP-delta may play a protective role in tumor cells and spur the growth and metastasis of tumor [41]. In our research, disposing LOVO cells with curcumin and irinotecan, we found that curcumin could suppress the expression of TRAP-delta while irinotecan raised it. In each treatment group, the various trend of TRAP-delta expression resembles Glutathione S-transferase Mu 5. So we speculate that after treated with irinotecan LOVO cells may launch their own protection mechanisms, increasing the expression of intracellular TRAP-delta and thereby decreasing injuries of irinotecan to their own. While by inhibiting the expression of TRAP-delta, curcumin not only achieves its own anti-tumor effect but also reduces the resistance of tumor cell to irinotecan and enhances the ability of irinotecan to kill LOVO cells, which may be another synergistic effect mechanism of these two drugs.

Protein disulfide-isomerase

Protein disulfide-isomerase, localized in endoplasmic reticulum as a chaperonin and with a molecular weight of 57 KDa, primarily plays the role of thiol peroxidase which catalyzes the formation, breakage and rearrangement of disulfide bond in cells [42]. It has been reported that the expression of protein disulfide-isomerase is activated in the endoplasmic reticulum stress and the unfolded protein response, the induction of which is also an anti-tumor strategy currently. In human ovarian cancer, prostate cancer, lung cancer, melanoma, glioma and acute lymphoma, Protein disulfide-isomerase is found at high expression levels, and inhibiting the activity of Protein disulfide-isomerase can promote apoptosis of tumor cells, suggesting that Protein disulfide-isomerase may be a choice target of anti-tumor drugs [43]. Studies have found that propynoic acid carbamoyl methyl

amides (PACMAs) can effectively suppress the activity of Protein disulfide-isomerase and lead to the accumulation of unfolded protein and misfolding protein, to cause endoplasmic reticulum stress and the unfolded protein response, which can promote the death of prostate cancer cells and also reverse the resistance of the prostate cancer cells to first-line chemotherapeutic drugs [44]. It suggests as well that a safe and effective anti-PDI inhibitor can serve as chemotherapy drug of prostate cancer and also may be used as second-line chemotherapy drug after the failure of first-line chemotherapy for prostate cancer. But whether it has a similar effect in colon cancer requests our continuing research. In addition, glutathioned protein disulfide-isomerase can cause unfolded protein response and interfere with the stability and function of ERα to promote the apoptosis of breast cancer cells [45]. But in hepatoma Hep3B cells, it has discovered that nelumbine could effectively induce apoptosis of Hep3B cells, and the action mechanism was also through the induction of endoplasmic reticulum stress in Hep3B cells. However, PDI expression in Hep3B cells is up-regulated after disposed by nelumbine, which indicates that while endoplasmic reticulum stress can be achieved by inhibiting the expression of PDI, this stress in cells occurs in multi-channel and one-sided up-regulation of the expression of PDI is not necessarily to inhibit endoplasmic reticulum stress [46]. In this study, we find that, compared with the negative control group, all the single-agent curcumin or irinotecan and mixed-administered group have no significant effect on the expression of PDI in LOVO cells while mixed-administered group slightly increases it. But in the sequential group, they can effectively suppress the expression of PDI. It is can be explained that the Sequential administration of curcumin and irinotecan may achieves to promote the apoptosis of LOVO cells, by inhibiting the expression of PDI in these cells and triggering endoplasmic reticulum stress and unfolded protein response, PDI may be a target of the sequence combination effect of these two drugs.

In short, based on our study results, we found that the combination of curcumin and irinotecan can indeed enhance the killing effect of irinotecan to LOVO cells. Relevant mechanism has been analyzed above, but there remain lots of deficiencies in this study. Up to date, we have just proved this effect phenomenon and relevant mechanism in LOVO cell, and our research work has not been carried out in other colon cancer cell lines and animal experiments. In the further study, we will continue to improve our research work in other colon cancer cell lines and animal experiments to provide more adequate experimental basis for the two drugs in joint application.

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Disclosure of conflict of interest

None.

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