Proteomic analysis of cell lines to identify the irinotecan resistance proteins

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Chemotherapeutic drug resistance is a frequent cause of treatment failure in colon cancer patients. Several mechanisms have been implicated in drug resistance. However, they are not sufficient to exhaustively account for this resistance emergence. In this study, two-dimensional gel electrophoresis (2-DE) and the PDQuest software analysis were applied to compare the differential expression of irinotecan-resistance-associated protein in human colon adenocarcinoma LoVo cells and irinotecan-resistant LoVo cells (LoVo/irinotecan). The differential protein dots were excised and analysed by ESI-Q-TOF mass spectrometry (MS). Fifteen proteins were identified, including eight proteins with decreased expression and seven proteins with increased expression. The identified known proteins included those that function in diverse biological processes such as cellular transcription, cell apoptosis, electron transport/redox regulation, cell proliferation/differentiation and retinol metabolism pathways. Identification of such proteins could allow improved understanding of the mechanisms leading to the acquisition of chemoresistance.

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1. Introduction

Colon cancer remains the third most common cancer in both men and women (Jemal *et al.* 2009). The mainstay of nonsurgical treatment for advanced colon cancer has been 5-fluorouracil (5-FU), and current chemotherapeutic regimens generally consist of 5-FU/leucovorin combined with newer agents, such as irinotecan or oxaliplatin (Kelly and Goldberg 2005; Goldberg 2006).

Irinotecan is a prodrug converted by carboxylesterases into its active form, SN38. SN38, which is similar to other camptothecin derivatives, exerts its cytotoxic activity through the inhibition of topoisomerase I that is a 100 kDa nuclear enzyme needed for replication and transcription. SN38 interferes with topoisomerase I function by forming stable ternary complexes at the DNA breakage points and stopping the topoisomerase-I-mediated relegation (Slatter *et al.* 1997; Pommier *et al.* 1998).

However, the success of chemotherapy is marred by the development of resistance towards most cytostatic drugs. Tumours can develop acquired drug resistance phenotypes by the synthesis of the P-glycoprotein (Pgp) or breast

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Abbreviations used: ACN, acetonitrile; AKR, aldo-keto reductase; AR1A1, aldose reductase; CBB, Coomassie brilliant blue; CFL1, Co-filin-1; CRYAB, alpha-crystallin B chain; 2-DE, two-dimensional gel electrophoresis; DMEM, Dulbecco's modified Eagle medium; ELISA, enzyme-linked immunosorbent assay; 5-FU, 5-fluorouracil; HSP27, heat shock protein 27; IEF, isoelectric focusing; IPG, immobilised pH gradient; LoVo cells, human colon adenocarcinoma; LoVo/irinotecan, irinotecan-resistant human colon adenocarcinoma MDR, multidrug resistance; MS, mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; Pgp, P-glycoprotein; PVDF, polyvinylidene difluoride; Q-TOF, quadrupole time-of-flight; TBST, Tris-buffered saline Tween-20; TFA, trifluoroacetic acid

cancer resistance protein (Ling 1997; Lage 2003) and other different ordinarily unknown mechanisms (Zeng *et al.* 2000; Lage 2003). Although several pathways have already been elucidated, the exact mechanisms that lead to chemoresistance are unknown for a large number of tumours.

The proteomic approach has resulted in many opportunities and challenges in identifying new tumour markers and therapeutic targets, and in understanding disease pathogenesis (Vimalachandran and Costello 2004). An ideal approach to study the development of chemoresistance is the analysis of global protein expression. High-resolution two-dimensional gel electrophoresis (2-DE) that includes fractionation of proteins according to charge and mass is ideally suited for this purpose, though the method has some limitations, such as its labour-intensiveness and the limited dynamic range of the proteins separated (Chen *et al.* 2005).

To accomplish this, the cell line LoVo was treated continuously, over 6 months, with stepwise-increasing concentrations of irinotecan to yield the chemoresistant cell line that was named LoVo/irinotecan. In order to find differences between the parental cell lines and their chemoresistant counterparts, phenotyping was accomplished using 2-DE and image analysis of the fractionated proteins was accomplished using the PDQuest system. Proteins of interest were excised and subjected to microsequencing. Fifteen proteins with different abundances in these two cell lines were identified. Expression of obviously altered aldehyde reductase was also verified by Western blot analysis. We believe that such identified proteins could allow improved understanding of the mechanisms that lead to the acquisition of chemoresistance.

2. Materials and methods

2.1 *Cell culture and selection of chemoresistant cell lines*

The human colon adenocarcinoma LoVo cell was purchased from ATCC (Rockville, MD, USA). Cells were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco, USA) containing 10% fetal calf serum (FCS) (Hyclone, USA), penicillin (100 U/ml) and streptomycin (10 mg/l) at 37°C in an atmosphere containing 5% CO_2. The chemoresistant cell line that was named LoVo/irinotecan was selected from the wild-type LoVo cell line by chronic exposure to irinotecan (Sigma Chemical Co., St. Louis, MO, USA) in intermittent dosage schedules at sufficient intervals to permit the expression of the resistance phenotypes. The cells were exposed to the drugs starting from 1×IC50 (inhibitor concentration that causes 50% inhibition), and the concentrations were escalated at an increasing rate of 50%, and then cultured finally at fixed concentrations over a level of 10×IC50 of the chemotherapeutics. The chemoresistant cell lines were established and subcultured twice per week for more than 6 months until stable cell lines were established. For 2-DE analysis, cells (80% confluence) were washed twice with phosphate buffered saline (PBS) before harvesting. The cells were stored at –80°C until use.

2.2 *Drug sensitivity assay*

Cultured cells were suspended in DMEM at 1×10^5 cells/ml. A sample of 100 μl of the cell suspension was seeded into a 96-well plate. After an overnight culture, cells were exposed to the irinotecan for 48 h. After incubation with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 0.5 mg/ml) for 4 h, the medium was removed and 150 μl of dimethylsulfoxide was added to dissolve formazan crystals. Absorbance was measured at 570 nm using an ELISA microplate reader (PerkinElmer, Wellesley, MA, USA). Cell survival rates were calculated according to the following formula: survival rate = (mean A570 of treated wells/mean A570 of untreated wells) × 100%. Finally, dose–effect curves of anticancer drugs were drawn on semilogarithm graphs, and IC50 values were determined.

2.3 *2-DE and image analysis*

The 2-DE was performed as described previously (Tong *et al*. 2007) with minor modification. Approximately 4×10^7 cells were lysed in 1 ml lysis buffer (7 *M* urea, 2 *M* thiourea, 4% 3-[(3-Cholanidopropyl)dimethylammonio]-1-propanesulfonate CHAPS, 100 m*M* dithiotheritol (DTT), 0.2% pH 3–10 ampholyte; Bio-Rad, USA) containing protease inhibitor cocktail 8340 (Sigma, St. Louis, MO, USA). Samples were then kept on ice and sonicated in six cycles of 15 s, each cycle consisting of a 5 s sonication followed by a 10 s break. After centrifugation at 15000 rpm for 1 h at 4°C, the supernatant was collected and the protein concentrations were determined using the DC protein assay kit (Bio-Rad). Protein samples (2 mg) were applied to immobilised pH gradient (IPG) strip (17 cm, pH 3–10 non linear [NL], Bio-Rad) using a passive rehydration method. After 12–16 h of rehydration, the strips were transferred to an isoelectric (IEF) cell (Bio-Rad). IEF was performed as follows: 250 V for 30 min, linear; 1000 V for 1 h, rapid; linear ramping to 10000 V for 5 h, and finally 10000 V for 4 h. The second dimension was performed using 12% SDS-PAGE at 30 mA constant current per gel after equilibration. The gels were stained using CBB R-250 (Merck, Germany) and scanned with a Bio-Rad GS-800 scanner. Three independent runs were made for each cell line to ensure accuracy of analyses. The maps were analysed by PDQuest software Version 6.1 (Bio-Rad). The quantity of each spot in a gel was normalized as a percentage of the total quantity of all spots in that gel and evaluated in terms of OD. For statistical analysis,

paired *t*-test was performed to compare data from the three repeated experiments. Only spots that showed consistent and significant differences (± over two-fold, $P < 0.05$) were selected for analysis with MS.

2.4 *In-gel digestion*

In-gel digestion of proteins was carried out using MS-grade Trypsin Gold (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, spots were cut out of the gel (1–2 mm diameter) using a razor blade, and destained twice with 100 m*M* NH_4HCO_3/50% ACN at 37°C for 45 min in each treatment. After drying, 200 ng trypsin was used per spot for 1 h. Then, 15 ml digestion buffer was added (40 m*M* NH_4HCO_3/10% ACN) to cover each gel and incubated overnight at 37°C Tryptic digests were extracted using MilliQ water initially, followed by two-times extraction with 50% ACN/5% TFA for 1 h each time. The combined extracts were dried in a vacuum concentrator at room temperature. The samples were then subjected to MS analysis.

2.5 *ESI-Q-TOF analysis and protein identification*

Mass spectra were acquired using a Q-TOF mass spectrometer (Micromass, Manchester, UK) fitted with an ESI source (Waters). Tryptic digests were dissolved in 18 μl 50% ACN. MS/MS was performed in a data-dependent mode in which the top-10 most abundant ions for each MS scan were selected for MS/MS analysis. Trypsin autolysis products and keratin-derived precursor ions were automatically excluded. The MS/MS data were acquired and processed using MassLynx software (Micromass) and MASCOT was used to search the database. Database searches were carried out using the following parameters: database, Swiss-Prot; taxonomy, homo sapiens; enzyme, trypsin; mass tolerance, ± 0.1 Da; MS/MS tolerance, ± 0.05 Da; and an allowance of one missed cleavage. Fixed modifications of cysteine carboamidomethylation, and variable modifications of methionine oxidation were allowed. The data format was selected as Micromass PKL and the instrument was selected as ESI-Q-TOF. Proteins with probability based MOWSE scores exceeding their threshold (P<0.05) were considered to be positively identified. To eliminate the redundancy of proteins appearing in the database under different names or accession numbers, the one-protein member with the highest MASCOT score, and belonging to *Homo sapiens*, was further selected from the relevant multiple-member protein family.

2.6 *Western blot*

LoVo and LoVo/irinotecan cell proteins were extracted in RIPA buffer (50 mM Tris-base, 1.0 m*M* EDTA, 150 m*M* NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 1 m*M* PMSF) and quantified by the DC protein assay kit (Bio-Rad). Samples were separated by 12% SDS-PAGE and transferred to PVDF membranes (Amersham Biosciences). The membranes were blocked overnight with PBS containing 0.1% Tween 20 in 5% skimmed milk at 4°C, and subsequently probed by the primary antibodies: rabbit-anti-AKR1A1 (diluted 1:1000 Santa Cruz Biotechnology, USA). Blots were incubated with the respective primary antibodies for 2 h at room temperature and washed three times in Tris-buffered saline Tween-20 (TBST). After that, the blots were incubated with secondary antibody (diluted 1:10 000) conjugated to HRP for 2 h at room temperature. Target proteins were detected by enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, USA). GAPDH was used as an internal control.

3. Results

3.1 *Biological characteristics of LoVo/irinotecan cells*

The IC50 values for irinotecan treatment in LoVo and LoVo/irinotecan cells were 5.07±1.34 μg/l and 79.24 ± 8.43 μg/l, respectively. The resistance of LoVo/irinotecan cells to irinotecan was 15.63-fold higher than that of LoVo cells, which meant irinotecan-resistant cell line was successfully established.

3.2 *2-DE profiling of the differentially expressed proteins between LoVo and LoVo/irinotecan cells*

The protein expression profile of LoVo and LoVo/irinotecan cells were examined by 2-DE. The experiments were repeated at least three times with reproducible results. A pair of representative 2-DE maps is shown in figure 1. The spot with a remarkable expression change is shown in figure 2 in an enlarged form. Fifteen proteins were successfully identified with good peptide coverage and significant scores using ESI-Q-TOF–MS in combination with a database search (table 1). Seven of the 15 proteins were up-regulated in LoVo/irinotecan cells and 8 were down-regulated. Among these spots, spot #10 was positively identified with high confidence (table 1). The average values of the MOWSE score and the number of unique peptides identified by MS/MS sequencing were 105 and 12, respectively. A representative MS map of spot #10 is shown in figure 3.

3.3 *Protein validation by western blot*

AKR1A1 was further validated by western blot. Consistent with the observations in 2-DE analysis, AKR1A1 was

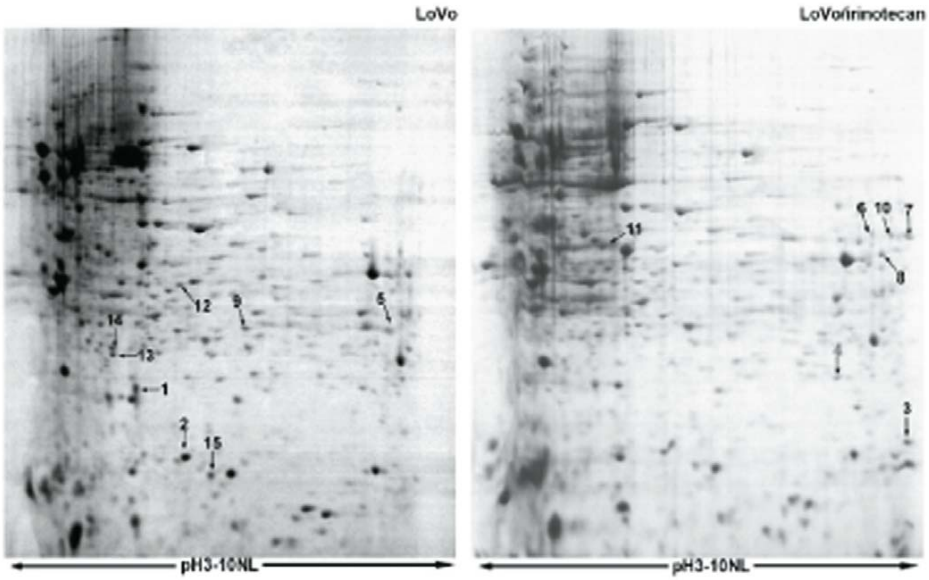

Figure 1. A representative map of the protein expression patterns between LoVo cells and irinotecan-resistant LoVo cells. Three independent 2-DE experiments were performed for the two cell lines to ensure the accuracy of analyses. The arrows indicate the 15 differentially expressed proteins.

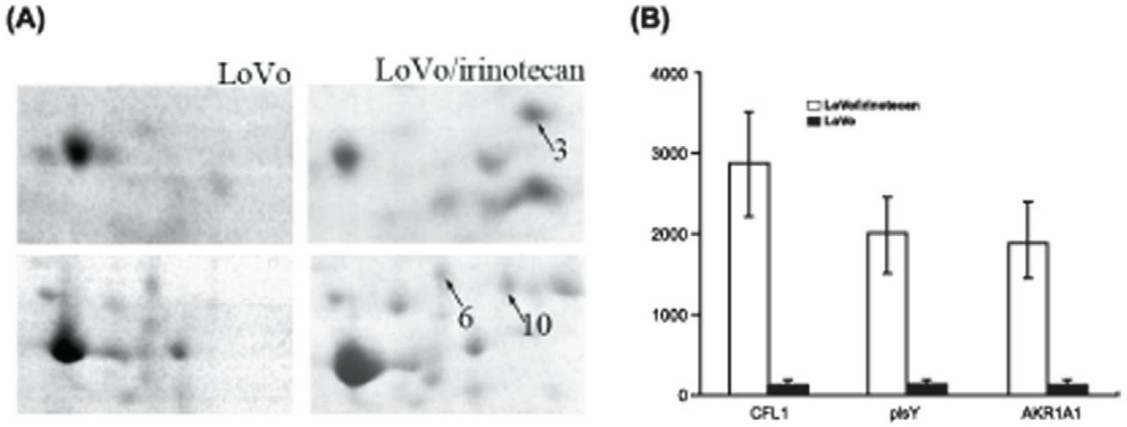

Figure 2. Enlargement of selected regions as examples showing spots with consistent expression variation in two-dimensional polyacrylamide gel electrophoresis (2-DE) gel. **(A)** Spots # 3, # 6 and # 10 were significantly up-regulated in LoVo/irinotecan cells compared with LoVo cells. **(B)** The quantity of each spot was determined as described. Data are shown as mean values ± SD.

up-regulated particularly in the LoVo/irinotecan cells compared with the LoVo cells (figure 4).

4. Discussion

Chemotherapy appears to be a useful tool in the management of advanced colon cancer, with several randomized trials demonstrating a modest but real benefit in prolonging survival and maintaining quality of life (Kelly and Goldberg 2005; Goldberg 2006). It may fail for various reasons. Among these, drug resistance is the most important one. During treatment, resistance towards anticancer drugs develops in approximately a third of all cases (Hütter and Sinha 2001). Identification of resistance-associated protein may provide new and specific targets for cancer therapy.

In this study, we established a human colon cancer cell line made resistant to irinotecan. The resistance of LoVo/irinotecan to irinotecan was 15.63-fold higher than that of LoVo cells. We identified 15 differentially expressed proteins

Table1. Identified proteins by MS/MS analysis

Spot No.	Protein description	Gene name	Function	Accession No.	Theoretical Mr/pI	Score	No. of peptides	Fold change
1	Cellular retinoic-acid-binding protein 1	*CRABP1*	Retinol metabolism	P29762	17566/5.31	705	20	↓ N/A
2	Stathmin	*STMN1*	Metabolism	P16949	15303/5.77	261	11	↓ 2.6±0.5
3	Cofilin-1	*CFL1*	Anti-apoptosis	P23528	18502/8.26	134	20	↑ N/A
4	Alpha-crystallin B chain	*CRYAB*	Anti-apoptosis	P02511	20158/7.76	519	18	↑ N/A
5	Triosephosphate isomerase	*TPI1*	Metabolism	P60174	26538/8.51	277	12	↓ 4.5±1.8
6	Glycerol-3-phosphate acyltransferase	*plsY*	Metabolism	Q2GLU7	21303/9.63	45	1	↑ N/A
7	Peptidyl-prolyl cis-trans isomerase A	*PPIA*	Metabolism	P62937	18012/7.82	64	3	↑ N/A
8	Fructose-bisphosphate aldolase A	*ALDOA*	Metabolism	P04075	39420/8.39	92	2	↑ 2.0±0.8
9	Peroxiredoxin-4	*PRDX4*	Electron transport/ Redox regulation	Q13162	30540/6.54	221	10	↓ 2.8±0.7
10	Aldehyde reductase	*AKR1A1*	Metabolism	P14550	36573/8.35	105	12	↑ N/A
11	Inorganic pyrophosphatase	*PPA1*	Metabolism	Q15181	32660/5.54	255	12	↑ 3.3±0.7
12	Serum albumin	*ALB*	Main protein of plasma	P02768	69367/8.34	87	2	↓ 2.3±0.4
13	Actin-like protein 6A	*ACTL6A*	Cellular transcription	O96019	47461/5.39	36	3	↓ 2.1±0.3
14	Beta-enolase	*ENO3*	Cell proliferation/ differentiation	P13929	46932/7.73	42	4	↓ N/A
15	Nucleoside diphosphate kinase A	*NME1*	Cell proliferation/ differentiation	P15531	17149/5.84	226	17	↓ 2.3±0.5

Upward arrows: Up-regulated in irinotecan-resistant LoVo cells.
Downward arrows: Down-regulated in irinotecan-resistant LoVo cells.

with 2-DE and mass spectrometry in the irinotecan-resistant cell line LoVo/irinotecan compared with its parental cell line LoVo. Some of these are proteins involved in metabolic enzymes, other proteins related to cell apoptosis and yet other proteins related to cellular transcription and so on. Among them, some of the proteins are associated with the development of tumour drug resistance, such as aldehyde reductase, whereas some might be new drug-resistance-associated proteins, such as retinoic-acid-binding protein I, and others have not been previously linked to drug resistance.

AKR1A1, previously designated as aldehyde reductase, belongs to the aldo-keto reductases (AKR) superfamily (Matsunaga *et al.* 2006; Jin and Penning 2007) and is a well-known cytosolic enzyme participating in chemotherapeutic drug carbonyl reduction (Ohara *et al.* 1995). In the previous study overexpression of AKR1A1 was followed by 4.5-fold increase in daunorubicin resistance of the tumour cells (Mariann *et al.* 2007). The carbonyl reduction takes place on the side-chain C-13 carbonyl moiety of daunorubicin, resulting in the formation of the secondary alcohol metabolites daunorubicinol (Forrest and Gonzalez 2000; Mordente *et al.* 2003). Daunorubicinol is not only significantly less potent than the parent drug in terms of inhibiting tumour cell growth *in vitro* (Olson *et al.* 1988; Schott and Robert 1989; Kuffel *et al.* 1992) but also is thought to be responsible for the cardiotoxicity observed upon chemotherapy (Li *et al.* 1991). The finding that overexpression of aldose reductase may contribute to doxorubicin resistance has also been reported before (Licata *et al.* 2000). In our study, AKR1A1 was found by 2-DE to be up-regulated in LoVo/irinotecan cells compared with LoVo

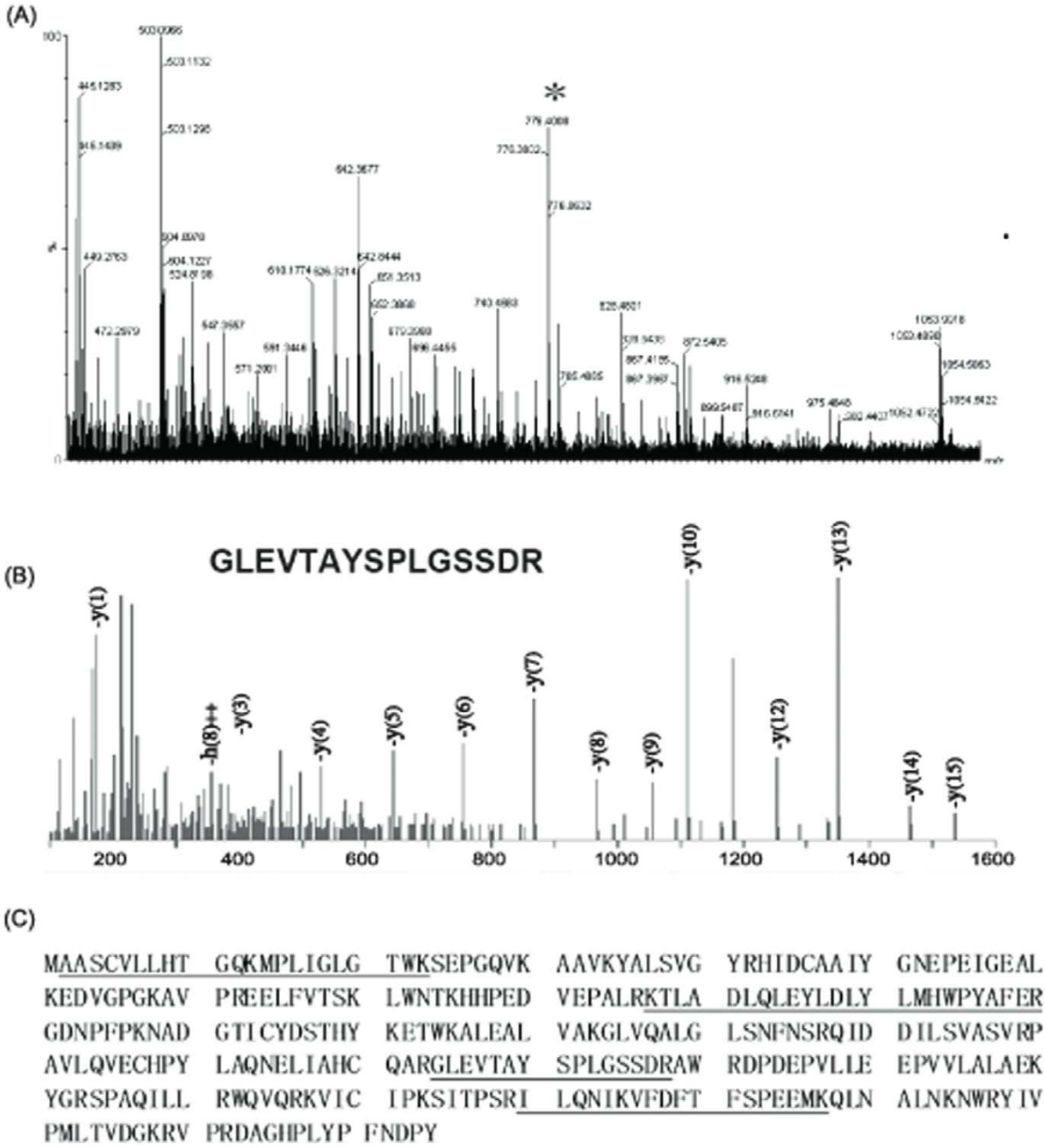

Figure 3. Results of AKR1A1 as the representative of protein identification using ESI-Q-TOF-MS/MS. **(A)** Mass spectrogram of tryptic peptides from spot #10. Four peptides with *m*/*z* values were subjected to mass spectrometry (MS)/MS analysis. **(B)** An example of an MS/MS spectrum of parent ion 776.4008. **(C)** Protein sequence of AKR1A1. The matched peptides are underlined.

cells. Western blot analysis showed that similar changes of expression levels of AKR1A1 in LoVo/irinotecan. Thus, AKR1A1 may play an important role in the development of acquired irinotecan resistance.

Apoptosis is of great importance in the understanding and treatment of cancer. It has been reported that genes that regulate apoptotic cell death may play an important role in determining the sensitivity of tumour cells to chemotherapy.

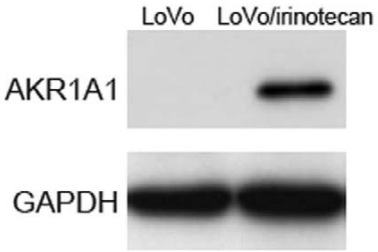

Figure 4. Western blot confirmation of the proteins AKR1A1 corresponding to the spots # 10. The images are the representative results of at least three independent experiments. As shown, expression of AKR1A1 was up-regulated in LoVo/irinotecan cells comparing with LoVo cells. GADPH was used as a loading control.

Resistance to apoptosis plays an important role in malignancies that are refractory to chemotherapy. Inhibitors of apoptosis can induce resistance to chemotherapeutic drugs (Perez 1998; Yu 1998). Interestingly, some of the up-regulated proteins identified in this study are anti-apoptosis proteins (including Cofilin-1 [CFL1] and alpha-crystallin B chain [CRYAB]). The gene *CRYAB* codes for a small heat shock protein shown to be overexpressed in a variety of neurological tumours and identified as a negative regulator of apoptosis (Judson *et al.* 2004; Watanabe *et al.* 2009). Moreover, CRYAB is related to the small heat shock protein Hsp27, another negative regulator of apoptosis (Arrigo *et al.* 2007; Judson *et al.* 2004). Both genes are mapped to chromosome 11q22.3-11q23.1, a region recently reported before to be involved in a reciprocal translocation with chromosome 22 in cells from recurrent tumours (Arrigo *et al.* 2007). Overexpression of the gene *CRYAB* was associated with carmustine resistant glioma cells (Judson *et al.* 2004). Cofilin 1, an actin depolymerising protein, can control actin-based motility by reversible phosphorylation. Human pancreatic carcinoma cell line EPP85-181P, a classical multidrug resistance (MDR) subline EPP85-181RDB selected in the presence of daunorubicin and an atypical (MDR) cell variant EPP85-181RNOV selected in the presence of mitoxantrone were analysed using 2-DE. After staining and image analysis spots of interest were isolated using preparative 2-DE and subjected to MS and microsequencing. Three proteins including E-FABP, stratifin and cofilin were overexpressed in chemoresistant cell lines. Cofilin was present in both MDR cell lines. E-FABP and stratifin was found to be overexpressed only in the mitoxantrone selected atypical MDR cell line (Sinha *et al.* 1999).

Cellular retinoic-acid-binding protein 1 was found obviously down-regulated in LoVo/irinotecan cells compared with the LoVo cells. This protein normally binds retinoic acid and is involved in the cellular signalling pathway of this compound, which inhibits cell growth (Flagiello *et al.* 1997; Tanaka *et al.* 2007). The significance of down-regulation of this protein remains to be verified and clarified.

In conclusion, accumulating data suggests that multiple complex mechanisms may be involved, simultaneously or complementarily, in the emergence and development of drug resistance in cancers. LoVo and LoVo/irinotecan cell lines are useful methods to elucidate the resistance mechanism in colon cancer. In our study, using proteomic approach, we identified 15 protein spots that have altered expression in the drug-resistant colon cancer cell line LoVo/irinotecan compared with the parental sensitive cell line LoVo, and some significant results were confirmed with Western blot. We believe that the data may be useful for further study of resistance mechanisms and screening of resistant biomarkers. Further studies are being carried out to validate the findings in other cell lines as well as in pathological specimens from patients, which can provide further information about the chemoresistant mechanisms.

Acknowledgements

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