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A Subcelluar Proteomic Investigation into Vincristine-Resistant Gastric Cancer Cell Line

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Abstract Multidrug resistance (MDR) is a major obstacle to successful cancer treatment. To understand the mechanism of MDR better, a subcelluar proteomics approach was used to compare the protein profile between vincristine-resistant human gastric cancer cell line SGC7901/VCR and its parental cell line SGC7901. After differential solubilization, the subfractionation proteins were separate by two-dimensional gel electrophoresis (2-DE), and the differential protein spots were identified by both MALDI-TOF-MS and ESI-Q-TOF-MS. Then the differential expressional levels of partial identified proteins were determined by Western blot analysis. Furthermore, one of the highly expressed proteins in SGC7901/VCR, Sorcin, associated with MDR was analyzed. In this study, the well-resolved, reproducible 2-DE patterns of subfractionation proteins from SGC7901/VCR and SGC7901 were established, and 30 differential proteins between the two cell lines were identified. The functional validation showed that the elevated sorcin expression could contribute considerably to the vincristine resistance in SGC7901/VCR. The 30 differentially expressed proteins could be divided into six groups based on their functions: calcium binding proteins, chaperones, metabolic enzymes, proteins relative to signal transduction, proteins involved in transcription and translation, and transportation proteins, and most of them might be new MDR associated proteins, which have not been detected previously. These data will be valuable for further to study the mechanisms of MDR in human gastric cancer. J. Cell. Biochem. 104: 1010–1021, 2008.

Key words: multidrug resistance; gastric cancer; two-dimensional gel electrophoresis; mass spectrometry; proteome analysis; subfractionation proteins isolation

Multidrug resistance (MDR) describes a phenomenon of cross-resistance of tumor cells to several structurally unrelated chemotherapeutic agents after exposure to a single cytotoxic drug. MDR is the main cause for chemotherapy failure of malignant tumors, including gastric cancer. Various mechanisms of cellular drug resistance that occur at a molecular level have been described and intensively studied over the past decades in vitro and in vivo. They include reduction of the intracellular accumulation of anti-cancer drugs by increasing drug efflux and/or decreasing drug uptake [e.g., P-glycoprotein [Gottesman et al., 2002], MDR related protein [Lee et al., 2004], and lung resistance related protein [Yeh et al., 2005]], alterations in drug targets [e.g., topoisomerase II [Stein et al., 2003]], and activation of detoxifying systems [e.g., glutathione/glutathione-*S* transferases [Kramer et al., 1988; Depeille et al., 2005]]. Increased repair of drug-induced DNA damage, blocked apoptosis, disruptions in signaling pathways, and alterations of factors involved in cell cycle control can also cause MDR [Hong et al., 2005; Lelong-Rebel and Cardarelli, 2005].

Gastric cancer is one of the most common malignant tumors throughout the world, particularly in Eastern Asian countries such as China, Korea, and Japan [Parkin, 2001]. Resistance to anti-cancer drugs is a major obstacle preventing the effective treatment of gastric

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cancer. The MDR mechanism in gastric cancer cells has been broadly explored, but it is still unclear. Multidrug-resistant gastric cancer cell line SGC7901/VCR, which derived from human gastric cancer cell line SGC7901 by stepwise selection in vitro using vincristine as an inducing reagent, and can also cross-resist to other anti-cancer drugs such as cisplatin, etoposide, mitomycin C, and 5-fluorouracil (5-FU) [Zhao et al., 2002], is a useful cell model for investigating the mechanism underlying MDR in gastric cancer. The proteomics approach provides us a new tool to study the pathogenesis of MDR of cancer cells. Two-dimensional electrophoresis (2-DE) is the principal step of proteomics and widely used in comparative studies of protein expression levels. However, one limitation of 2-DE of total proteins is the inability to detect low abundance proteins, usually because of interference by major protein species [Sakai et al., 2003]. Much effort is therefore directed at detecting low abundance proteins. Today, very effective protocols exist for pre-fractionating the entire cell lysate, and analyzing it in narrow pH gradients, greatly enhancing detection of rare proteins. In the present study, our work is in progress in this direction. 2-DE was used to separate the proteins after differential solubilization of SGC7901/VCR and SGC7901 cell lysates. Pilot runs with pH 3-10 IPG strips revealed most of the proteins focused within the pH 4–7 range, so IPG strips with narrower pH range of 4–7 were used to achieve a better separation of these protein after subfractionation proteins isolation. In this work, the 2-DE maps of SGC7901/VCR and SGC7901 were established using subfractionation proteins, and 30 differential expressional proteins were identified, and most of them might be new MDR associated proteins, which have not been detected previously. Furthermore, the effect of Sorcin, one of the highly expressed proteins in SGC7901/VCR, on the development of MDR of SGC7901/VCR was analyzed. The results presented here will no doubt provide new clues to further study the mechanisms of MDR in human gastric cancer.

MATERIALS AND METHODS

Instruments and Software

IPGphor, Ettan DALT II System, ImageScanner (maximum resolution 9,600 × 9,600 dpi) (Amersham Biosciences, Stockholm, Sweden), ESI-Q-TOF Mass Spectrometry (Micromass, Manchester, UK), PDQuest system (Bio-Rad laboratories, Hercules, CA), Mascot Distiller and Mascot Database Search engine, and Statistical Package For Social Science (SPSS for windows, Version 10.01, USA) were used.

Cell Lines

Vincristine-resistant human gastric carcinoma cell line SGC7901/VCR and its parental cell line SGC7901 were a gift from Dr. DM Fan (The Fourth Military Medical University, China). SGC7901 was cultured with RPMI1640 medium containing 10% fetal calf serum (Gibico BRL, Grand Island, NY). To maintain biologic characteristics of MDR, SGC7901/VCR was cultured with RPMI1640 medium containing 10% fetal calf serum and 1 mg/L vincristine (Sigma–Aldrich, St. louis, MO).

Reagents

Immobiline pH-gradient (IPG) DryStrips (pH 4–7 L, 24 cm), IPG buffer (pH 4–7), DryStrip cover fluids, thiourea, urea, CHAPS, DTT, Pharmalyte (pH 4–7), bromophenol blue, Bis, TEMED, commassie brilliant blue G-250, low molecular weight marker, Tris-base, SDS, glycine, PVDF membrane, goat anti-mouse, goat anti-rabbit, or rabbit anti-goat IgG-conjugated with horseradish peroxidase, and the enhanced chemiluminescence (ECL) system were purchased from Amersham Biosciences. Sequencing-grade modified trypsin was obtained from Promega. ZipTip C^{18} columns were obtained from Millipore, Bedford, MA. Monoclonal or polyclonal antibodies against GRP78, Annexin1, Sorcin, EEF2 and α-Tublin, and Sorcin siRNA kit were from Santa Cruz Biotechnology, Santa Czuz, CA. Mercaptoethanol, iodoacetamide, α-cyano-4-hydroxycinnamic (CCA), and HCl were from Sigma–Aldrich. ProteoExtract™ Partial Mammalian Proteome Extraction Kit was purchased from Calbiochem, San Diego, CA. PAGE (12%) resolving gel was cast with a Ettan DALT II Gel Caster. All buffers were prepared with Milli-Q water.

Subfractionation Proteins Isolation

About 2×10^8 cells at 80% confluency wereharvested, and proteins were sequentially extracted with the ProteoExtract™ Partial Mammalian Proteome Extraction Kit based on differential solubility according to the manufacturers instructions with a small alteration.

Briefly, Protease Inhibitor Cocktail (Calbiochem) was added to protein extraction reagents prior to extraction at a final concentration of 10 μl/ml to reduce protein degradation. Cells were homogenized in Extraction Reagent 1 (Imidazole and Sucrose), and then the mixture was centrifuged at 28,000 rpm for 15 min at 4°C to separate most soluble proteins. The most soluble proteins in supernatants were precipitated using TCA/acetone as previously described [Yuan et al., 2002]. The original pellet (insoluble protein fraction) was washed in Extraction Reagent 1 until in cell sample of interest no significant changes in absorption measured at 280 nm between washing steps, and then resuspended in Extraction Reagent 2 (Urea, Thiourea, Sulfobetain, Non-ionic Detergent, and DTT). The concentration of the subfractionation proteins was determined using 2D Quantification kit (Amersham Biosciences).

2-DE

2-DE was performed as described by the manufacturer (Amersham Biosciences). Protein samples (500 μg for preparative gels) were diluted to 450 μl with rehydration solution [7 mol/L urea, 2 mol/L thiourea, 0.2% DTT, 0.5%(v/v) pH4–7 IPG buffer, and trace bromophenol blue], and applied to IPG strips (pH 4–7 L, 240 mm × 3 mm × 0.5 mm) by 24 h rehydration at 30 V. Proteins were focused successively for 1 h at 500 V, 1 h at 1,000 V and 10 h at 8,000 V to give a total of 80kVh on an IPGphor. Focused IPG strips were equilibrated for 15 min in a solution (6 mol/L urea, 2% SDS, 30% glycerol, 50 mmol/L Tris–HCl, pH 8.8 and 1% DTT), and then for an additional 15 min in the same solution except that DTT was replaced by 2.5% iodoacetamide. After equilibration, second-dimension sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on Ettan DALT II system. Then, the Blue Silver staining method (a modified Neuhoff's colloidal coomassie blue G-250 stain) was used to visualize the protein spots in the 2-DE gels [Candiano et al., 2004].

Image Analysis and Spot Identification

The stained 2-DE gels were scanned with MagicScan software on Imagescanner and analyzed using PDQuest system according to the protocols provided by the manufacturer. To minimize the contribution of experimental variations, three separate gels were prepared for each subcellular preparation. The densities of the spots were determined after normalization based on the total spot volumes on the gel. The protein spots from different gels were matched. The protein spots with significant changes in densities (paired t-test, $P < 0.05$) in a consistent direction (increase or decrease) were considered to be different, and selected for further identification.

Protein Identification

All the differential protein spots were excised from preparative gels using punch, and transferred to a 1.5 ml siliconized Eppendorf tubes. One protein-free gel piece was treated in parallel as a negative control. The gel-spots were washed three times with two-distillation water, which was then removed. The fresh solution containing 100 mmol/L NH_4HCO_3 in 50% acetonitrile was used to decolor. The gel pieces were then dried in a vacuum centrifuge. The dried gel-pieces were incubated in 10 μl digestion solution consisted of 40 mmol/L NH_4HCO_3 in 9% acetonitrile solution, and 20 μg/ml proteomics grade trypsin for 10–12 h at 37°C. The tryptic peptide mixture was extracted and purified with Millipore ZipTip C^{18} column. The purified tryptic peptide mixture was mixed with CCA matrix solution, and vortexed gently. A volume (1 μl) of the mixture containing CCA matrix was loaded on a stainless steel plate and air-dried, then were analyzed with Voyager System DE-STR 4307 MALDI-TOF Mass Spectrometer (ABI). The standard peptide mixture was spotted at the same time to correct the machine. The parameters of MALDI-TOF were set up as follows: positive ion-reflector mode, accelerating voltage 20 kV, grid voltage 64.5%, mirror voltage ratio 1.12, N_2 laser wavelength 337 nm, pulse width 3 ns, the number of laser shots 50, acquisition mass range 500–3,000 Da, delay 100 ns, and vacuum degree 4×10^{-7} Torr. The differential protein spots identified by MALDI-TOF-MS were also subjected to analysis of ESI-Q-TOF-MS (Micromass). The samples were loaded on to a pre-column (320 μm × 50 mm, 5 μm C18 silica beads, Waters) at 30 μl/min flow rates for concentrations and fast desalting through a Waters CapLC autosampler, then eluted to the reversed-phase column (75 μm × 150 mm, 5 μm, 100 Å, LC Packing) at a flow rate of 200 μl/min after flow splitting for separation. MS/MS

spectra were performed in data-depended mode in which up to four precursor ions above an intensity threshold of seven counts/second (cps) were selected for MS/MS analysis from each survey "scan." The nanospray parameters were 3,000 V for capillary voltage, 45 V for cone voltage, 80°C for source temperature, and 15 psi collision gas back pressure.

Database Analysis

In PMF map database searching, Mascot Distiller was used to get the monoisotopic peak list from the raw mass spectrometry files. The monoisotopic peak list was used to search the Swiss-Prot or NCBInr database with Mascot search engine (http://www.matrixscience.com/). The searching parameter was set up as follows: the taxonomy was selected as all entries or homo sapiens; the mass tolerance was ±50 ppm; The missed cleavage sites were allowed up to 1; the fixed modifications were selected as carboxymethyl (cysteine); the variable modification was selected as oxidation (methylation) or none.

In tandem mass spectrometry data database query, the PKL format file that generated from MS/MS was imported into Mascot search engine. The searching parameter was set up as follows: the taxonomy was selected as all entries or homo sapiens; the mass tolerance was ±0.5 Da; the MS/MS tolerance was ±0.3 Da; The missed cleavage sites were allowed up to 1; the fixed modifications were selected as carboxymethyl (cysteine); the variable modification was selected as oxidation (methylation) or none; the data format was selected as Micromass PKL format; and the instrument was selected as ESI-Q-TOF.

Western Blot Analysis

The cells were collected from flasks, and washed with cold PBS for three times, then were lyzed at 4°C for 30 min in a lysis buffer (50 mmol/L Tris, pH7.4, 100 mmol/L $NaCl_2$, 1 mmol/L $MgCl_2$, 2.5 mmol/L Na_3VO_4, 1 mmol/L PMSF, 2.5 mmol/L EDTA, 0.5% Triton X-100, 0.5% NP-40, 5 μg/ml of aprotinin, pepstatin A, and leupeptin). The lysates were centrifuged at 11,000 rpm for 15 min at 4°C. Protein concentration was determined using the protein assay kit (Bio-Rad laboratories). The protein samples (about 20 μg) were separated by SDS–PAGE. After SDS–PAGE electrophoresis, proteins were transferred to PVDF membranes. The membranes were blocked overnight at 4°C with 5% nonfat dry milk in TBS-T buffer (20 mmol/L Tris, pH7.6, 100 mmol/L $NaCl_2$, 0.5% Tween-20), followed by 3h of incubation with the primary antibody (1:1,500–1:2,000 dilution) in TBS-T buffer containing 5% nonfat dry milk at room temperature. After washing three times with TBS-T buffer, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG, goat anti-rabbit IgG or rabbit anti-goat IgG as a secondary antibody (1:3,000 dilution) for 1h at room temperature. After the membranes were washed three times in TBS-T buffer, the reactions were visualized with ECL detection system. All of the Western blot analyses were repeated at least three times.

Administration of Sorcin siRNA to Cells

The cells were transfected with Sorcin siRNA or control siRNA (Santa Cruz Biotechnology) according to the siRNA transfection protocol provided by the manufacturer. Briefly, the day before transfection, SGC7901/VCR cells were plated into 6-well plates and 96-well plates at the density of 10^5 cells /ml medium, respectively. When the cells were 60–80% confluent, they were transfected with 10 nmol/L of Sorcin siRNA or control siRNA after a preincubation for 20 min with siRNA transfection reagent in siRNA transfection medium (Santa Cruz Biotechnology). Four hours after the beginning of the transfection, the medium was replaced with RPMI1640 medium containing 10% fetal calf serum, and continued to culture the cells for additional 44 h. At the end of the transfection they were incubated with different concentrations of vincristine. After 24-h incubation, sorcin expression level was determined by Western blot analysis described above, and the cell viability of SGC7901/VCR was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay described previously [Plumb et al., 1989].

Transfection of the Full-Length Sorcin Gene to Cells

The full-length sorcin gene was amplified from cDNA synthesized by RT-PCR using the total RNA extracted from cells SGC7901/VCR as the template. The following primers were used for PCR: forward: 5′-AAAAAGCTTATGG-CGTACCCGGGGCAT -3′ (*Hin*dIII site underlined); reverse: 5′-GGGTCTAGATTAAACACT-CATGACACATTG-3′ (*Xba*I site underlined) [Zhou et al., 2006]. The purified PCR products

were digested with *Hin*dIII and *Xba*I, and then cloned into the pcDNA3.0 vector (Invitrogen Life Technologies, Grand Island, NY) followed by sequence confirmation by DNA sequencing analysis. SGC7901 cells were transfected with pcDNA3.0/Sorcin or pcDNA3.0/control vector using lipofectamine2000 following the manufacturer's protocol (Invitrogen Life Technologies). Briefly, SGC7901 cells were seeded into 6-well plates, and when the cells were 90% confluent, they were transfected with 4 μg pcDNA3.0/Sorcin or pcDNA3.0/control vector. After 48 h incubation, the transfected SGC7901 cells were seeded in each well of 96-well plates and exposed to different concentrations of vincristine for additional 24 h to investigate their sensitivity to vincristine. The sorcin expression level in SGC7901 transfectants was determined by Western blot analysis, and the cell viability was examined using MTT assay described above.

Flow Cytometric Analysis of Apoptotic Cells

Vincristine-induced apoptosis was determined with flow cytometry according to the method previously described [Lau et al., 2004]. Briefly, SGC7901/VCR cells transfected with 10 nmol/L sorcin siRNA or SGC7901 cells transfected with pcDNA3.0/Sorcin, 48 h after transfection incubated with 1 mg/L vincristine for additional 24 h as described above. Then cells were harvested, washed with PBS, resuspended in FACS buffer containing RNAase A (0.2 μg/ml, Sigma) and propidium iodide (20 μg/ml, Sigma) and incubated at 37°C for 30 min. The stained cells were analyzed with a FACStar Plus flow cytometer. For each sample 1×10^6 cells were analyzed, providing a solid statistical basis for the determination of the percentage of apoptotic cells in each treatment using the Lysis II software program.

Statistical Analysis

The data were expressed as mean ± SE, and analyzed with the Student's *t*-test between two groups. It was considered statistically significant if *P*-value was less than 0.05.

RESULTS

Protein Patterns Following Differential Extraction

One limitation of 2-DE is the inability to detect low abundance proteins because of interference by major protein species. We therefore analyzed SGC7901/VCR and SGC7901 separated into two solubility fractions, using sequential extraction with the ProteoExtract™ Partial Mammalian Proteome Extraction Kit. One fraction contained proteins soluble in Extraction Reagent 1(Imidazole and Sucrose), and a second contained those proteins insoluble in Extraction Reagent 1, but soluble in Extraction Reagent2 (Urea, Thiourea, Sulfobetain, Non-ionic Detergent, and DTT). Image analysis using PDquest indicated about a total of 2,100 spots in the two gels of the sequential extraction, which suggested that after sequential extraction more protein spots could be detected as compared to the one-step extraction of total proteins. The representative 2-DE maps of fractions from SGC7901/VCR and SGC7901 cells following the sequential extraction procedure were shown in Figure 1. Compared with SGC7901, in the most soluble fraction 1, 22 spots were significantly modulated, and in the low soluble fraction 2, 17 spots were significantly modulated in SGC7901/VCR. A total of 39 differential expression proteins spots between the two cell lines were marked with arrows in Figure 1A,B.

MS Identification of the Differential Expression Proteins

All 39 protein differential spots were excised from the gels, digested with trypsin, and subsequently analyzed by MALDI-TOF-MS in combination with database searching. As a result, a total of 35 spots representing 30 non-redundant proteins were identified (Fig. 1 and Table I, spots 1–32, spots 35–36, and 38), of which nine proteins were up-regulated and 21 proteins were down-regulated. Due to the variations in sample preparation for 2-DE, the results generated from this subcellular fractionation protocol increase the chance to discover more less abundant and low solubility proteins, such as N-myc downstream regulated gene 1, etc. To confirm the protein identification, proteins were further analyzed by ESI-Q-TOF-MS and the results were consistent with that obtained by MALDI-TOF MS analysis. As shown in the 2-DE maps in Figure 1A,B, spot 16, 18, and 35 was significantly up-regulated in SGC7901/VCR and were identified as HSP27 (Fig. 2A,B).

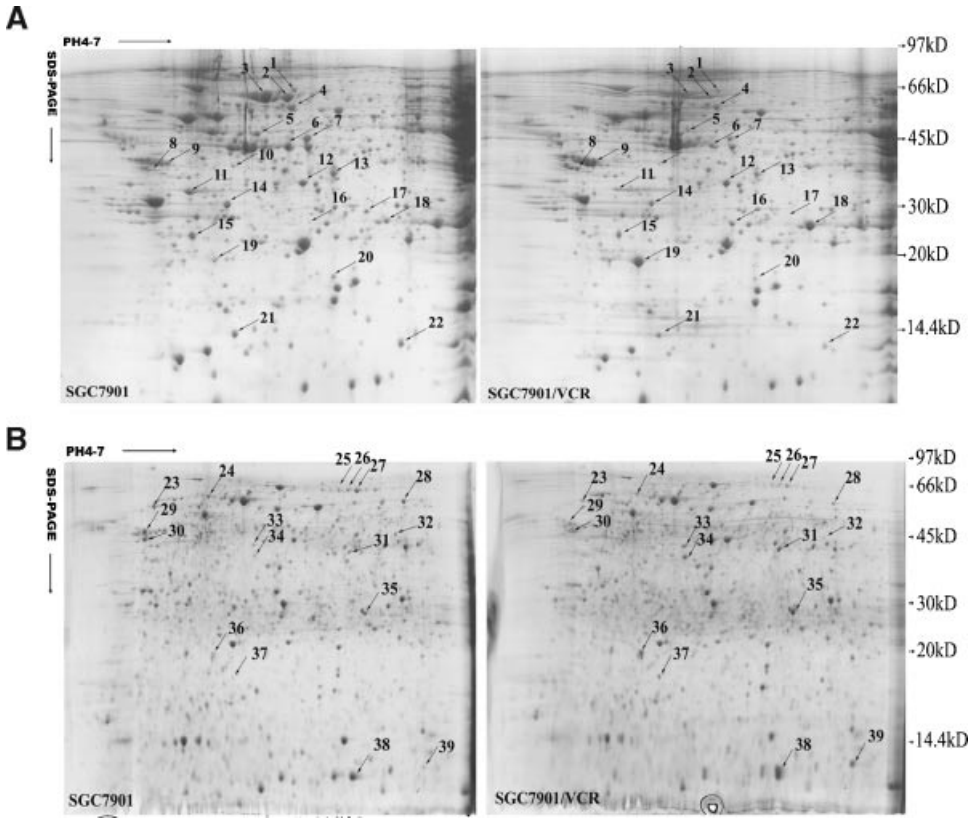


Fig. 1. Representative 2-DE maps showing differentially expressed proteins between SGC7901/VCR and SGC7901 following differential extraction. **A**: A 2-DE map of differential extraction fraction 1. **B**: A 2-DE map of differential extraction fraction 2. About 500 μg protein samples were separated on pH 4–7 nonlinear gradient IPG strips and 12.5% SDS–PAGE gels. Thirty-nine differential expression proteins spots between the two cell lines were marked with arrows in the maps.

Validation of Differential Expression Proteins

In order to confirm the differential expression levels of the proteins identified by comparative proteomics approach, the expressional levels of the four proteins with commercially available antibodies in SGC7901/VCR and SGC7901 were measured by Western blot analysis. Figure 3A showed a representative Western blot analysis result of Annexin1, GRP78, EEF2, and Sorcin expression in the two cell lines. Compared with SGC7901, vincristine-resistant SGC7901/VCR had an obvious up-regulation of Annexin1 and sorcin, and a marked down-regulation of GRP78 and EEF2, which were identical with the protein level changes observed in 2-DE gels (Fig. 3B).

The Association of Sorcin With MDR

To study the functional role of Sorcin up-regulation in SGC7901/VCR, SGC7901/VCR cells were transfected with Sorcin siRNA. Firstly, siRNA-induced inhibition of Sorcin expression was determined by Western blot analysis. As shown in Figure 4A, transfection of SGC7901/VCR cells with Sorcin siRNA significantly reduced Sorcin protein levels, whereas Sorcin protein expressions were not significantly suppressed by control siRNA. We next evaluated the effect of Sorcin siRNA transfection on the vincristine chemosensitivity in SGC7901/VCR. SGC7901/VCR cells were treated with Sorcin siRNA for 2 days, and for additional 24 h incubation with different concentrations of vincristine. And then the cell viability of SGC7901/VCR was examined using MTT assay. As Figure 4B shown, transfection of SGC7901/VCR cells with Sorcin siRNA could enhance vincristine chemosensitivity, with the significant decrease of the cell viability. Finally, induction of apoptosis by Sorcin siRNA was demonstrated by flow cytometry. After treated for 24 h with 1mg/L vincristine, the fraction of cells undergoing apoptosis was significantly

TABLE I. MALDI-TOF-MS and ESI-Q-TOF-MS Identified Proteins in SGC7901/VCR Versus SGC7901 Comparison

Spot	Gene name	Accession number[a]	Protein name	Theoretical Mr/pI	Expression in SGC7901/VCR	Function
1	HSPA9B	Q6GU03	Heat shock 70 kDa protein 9B	74,093/6.04	1.8-fold ↓[b]	Chaperone
2	HSC70	P11142	Heat shock cognate 71 kDa protein	71,082/5.37	2.4-fold ↓	Chaperone
3	HSPA8	Q96H53	HSPA8 protein	64,804/5.36	2.1-fold ↓	Chaperone
4	CCT5	P48643	T-complex protein 1, epsilon subunit	60,100/5.45	2.0-fold ↓	Chaperone
5	HNRPF	P52597	Heterogeneous nuclear ribonucleoprotein F	45,985/5.38	2.7-fold ↓	Chaperone
6	CKB	P12277	Creatine kinase B-type	42,933/5.34	2.2-fold ↓	Metabolism
7	NDRG1	Q92597	N-myc downstream regulated gene 1	43,264/5.49	2.1-fold ↓	Signal transduction
8	NPM1	P06748	Nucleophosmin	32,611/4.67	1.7-fold ↑[b]	Transportation
9	ANX1	P04083	Annexin1	38,787/6.64	2.4-fold ↑	Calcium-binding
10	SEC13L1	Q5BJF0	SEC13-like 1, isoform b	36,031/5.22	2.0-fold ↓	Metabolism
11	ANX5	P08758	Annexin5	35,839/4.98	1.8-fold ↓	Calcium-binding
12	PP	Q15181	Inorganic pyrophosphatase	35,962/5.95	1.8-fold ↑	Metabolism
13	LDHB	P07195	L-lactate dehydrogenase B chain	36,484/5.72	2.1-fold ↓	Metabolism
14	CLIC1	O00299	Chloride intracellular channel protein1	27,248/5.09	2.3-fold ↓	Signal transduction
15	GLO1	Q04760	Glyoxalase I	20,995/5.12	1.9-fold ↑	Metabolism
16,18,35	HSP27	Q96EI7	Heat shock protein 27	22,427/7.83	3.2,3.8,2.4-fold ↑	Chaperone
17	SET	Q5VXV1	SET translocation	33,469/4.23	5.8-fold ↓	Chaperone
19,36	SRI	Q30626	Sorcin	20,617/5.11	4.2,2.8-fold ↑	Calcium-binding
20	DUT	P33316	dUTP pyrophosphatase	17,908/6.15	1.8-fold ↓	Metabolism
21	EIF5A	P63241	Eukaryotic translation initiation factor 5A	17,049/5.08	1.7-fold ↑	Signal transduction
22	FABP3	P05413	Fatty acid-binding protein	14,775/6.34	2.0-fold ↓	Transportation
23	TRA1	Q59FC6	Tumor rejection antigen (Gp96) 1 variant	66,140/5.08	1.9-fold ↓	Chaperone
24	NUC	Q02818	Nucleobindin-1	53,846/5.15	1.8-fold ↓	Calcium-binding
25,26,27	ALPPL2	P10696	Alkaline phosphatase	57,706/6.03	1.9,1.8,1.8-fold ↓	Metabolism
28	OXCT1	P55809	3-ketoacid-coenzyme A transferase 1	56,578/7.14	2.1-fold ↓	Metabolism
29	GRP78	P11021	78 kDa glucose-regulated protein	72,402/5.07	2.2-fold ↓	Chaperone
30	RCN1	Q15293	Reticulocalbin-1	38,866/4.86	2.0-fold ↓	Calcium-binding
31	LMNA	P02545	Lamin A/C	53,219/6.13	2.1-fold ↑	Signal transduction
32	EEF2	P13639	Elongation factor 2	30,212/6.20	2.4-fold ↓	Transcription & translation
38	S100A11	P31949	S100 calcium-binding protein A11	11,847/6.56	1.8-fold ↑	Calcium-binding

[a]Swiss-Prot accession number.
[b]↑Showed that spot intensity increased in SGC7901/VCR in comparison to SGC7901; ↓showed that spot intensity decreased in SGC7901/VCR in comparison to SGC7901 (paired t-test, $P < 0.05$).

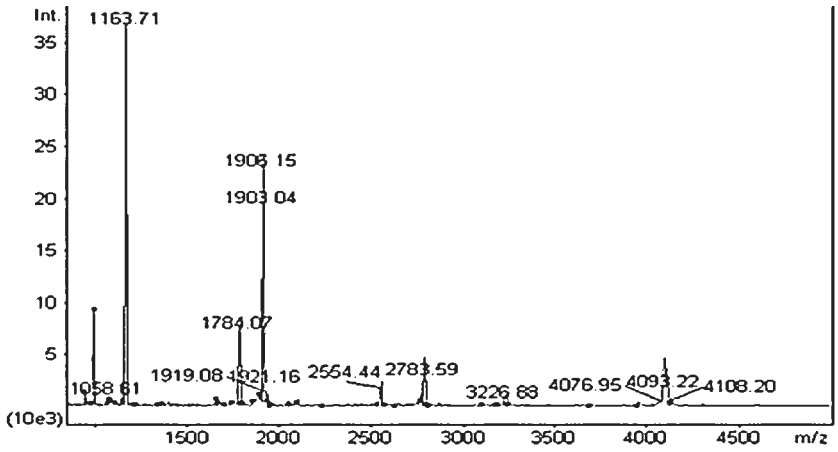
higher in SGC7901/VCR cells transfected with Sorcin siRNA than control siRNA (Fig. 4C), which clearly demonstrated that Sorcin siRNA could enhance vincristine chemosensitivity.

To further determine if the increased expression of sorcin potentially contributes to drug resistance, we also transfected the full-length Sorcin gene to SGC7901 cells. As shown in Figure 5A, after 48 h transfection the sorcin protein level increased in the SGC7901/Sorcin cells compared with the control SGC 7901/Vector cells. As shown in Figure 5B,C, it was obvious that the overexpression of sorcin caused resistance to vincristine.

DISCUSSION

In this study, we used proteomics approach to investigate MDR of vincristine-resistant cell line SGC7901/VCR after subfractionation protein isolation. We demonstrated that 30 proteins exhibited the changes of expression level in the vincristine-resistant cell line SGC7901/VCR compared with its counterpart SGC7901. The differentially expressed proteins could be divided into six groups based on their functions using information from Swiss-Prot and NCBInr websites: calcium binding proteins, chaperones, metabolic enzymes, proteins relative to signal

A



Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
5 - 12	987.6570	986.6497	986.6025	0.0472	1	R.RVPFSLLR.G
13 - 27	1903.0430	1902.0357	1901.8597	0.1760	1	R.GPSWDPFRSWFPHSR.L
28 - 37	1163.7110	1162.7037	1162.6134	0.0903	0	R.LFDQAFGLPR.L
38 - 75	4093.2220	4092.2147	4092.0533	0.1615	0	R.LPEEWSQWLGGSSWPGYVRPLPPAAIESPAVAAPAYSR.A
80 - 89	1075.6520	1074.6447	1074.5669	0.0778	0	R.QLSSGVSEIR.H
80 - 94	1655.8980	1654.8907	1654.8387	0.0521	1	R.QLSSGVSEIRHTADR.W
97 - 112	1784.0680	1783.0607	1782.9152	0.1456	0	R.VSLDVNHFAPDELTVK.T
113 - 123	1146.6910	1145.6837	1145.6291	0.0546	1	K.TKDGVVEITGK.H
128 - 136	1104.6160	1103.6087	1103.4996	0.1092	0	R.QDEHGYISR.C
141 - 171	3226.8840	3225.8767	3225.6478	0.2289	1	R.KYTLPPGVDPTQVSSSLSPEGTLTVEAPMPK.L
141 - 171	3242.8770	3241.8697	3241.6427	0.2270	1	R.KYTLPPGVDPTQVSSSLSPEGTLTVEAPMPK.L Oxidation (M)
142 - 171	3098.7890	3097.7817	3097.5529	0.2289	0	K.YTLPPGVDPTQVSSSLSPEGTLTVEAPMPK.L
172 - 188	1906.1480	1905.1407	1904.9843	0.1564	0	K.LATQSNEITIPVTFESR.A
189 - 198	941.5100	940.5027	940.4977	0.0050	0	R.AQLGGPEAAK.S

B

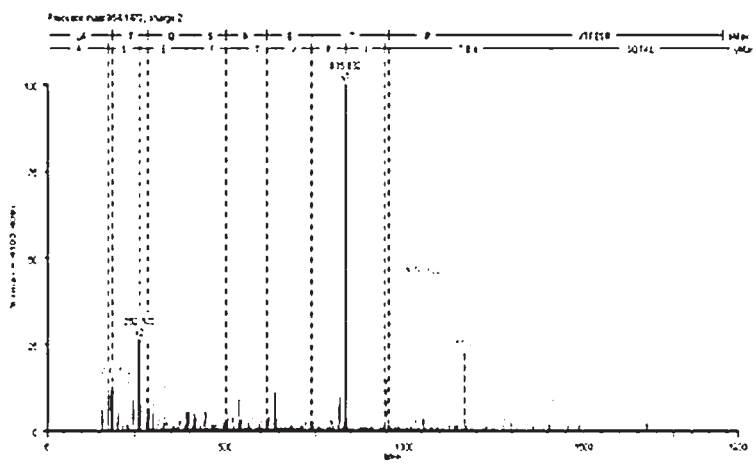


Fig. 2. MALDI-TOF-MS and ESI-Q-TOF-MS analysis of a representative differential protein HSP27. **A**: Identification of HSP27 by MALDI-TOF-MS after trypsin digestion of the protein according to the matched peaks. **B**: The amino acid sequence of a doubly charged peptide with *m/z* 954.1470 was identified as LATQSNEITIPVTFESR from mass differences in the y-fragment ions series by ESI-Q-TOF-MS, and matched with residues 172–188 of HSP27.

transduction, proteins involved in transcription and translation, and transportation proteins, some of which may be associated with MDR of human gastric carcinoma cell line SGC7901/VCR.

The expressions of six calcium-binding proteins obviously changed in SGC7901/VCR. Soluble resistance-related calcium-binding protein (sorcin), which has four typical "E–F hand" structures of calcium-binding sites, was highly expressed in vincristine-resistant SGC7901/VCR. The exact role and the underlying mechanisms of sorcin in drug resistance remain elusive. Most of proteomic studies in a number of other MDR cell lines have not always shown the high expression of sorcin. Even in those

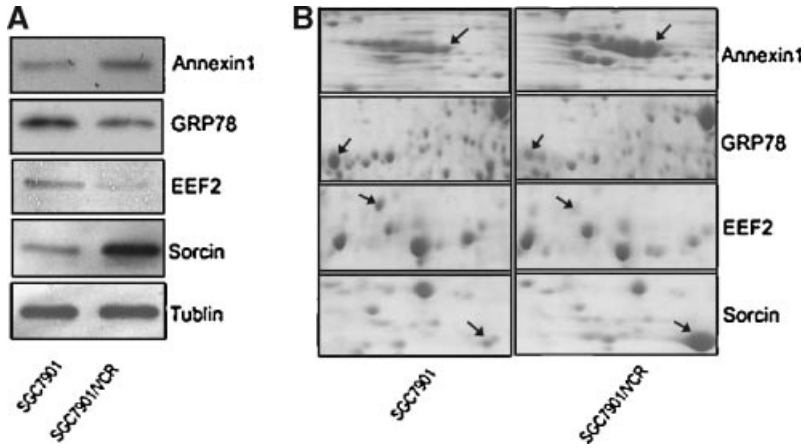
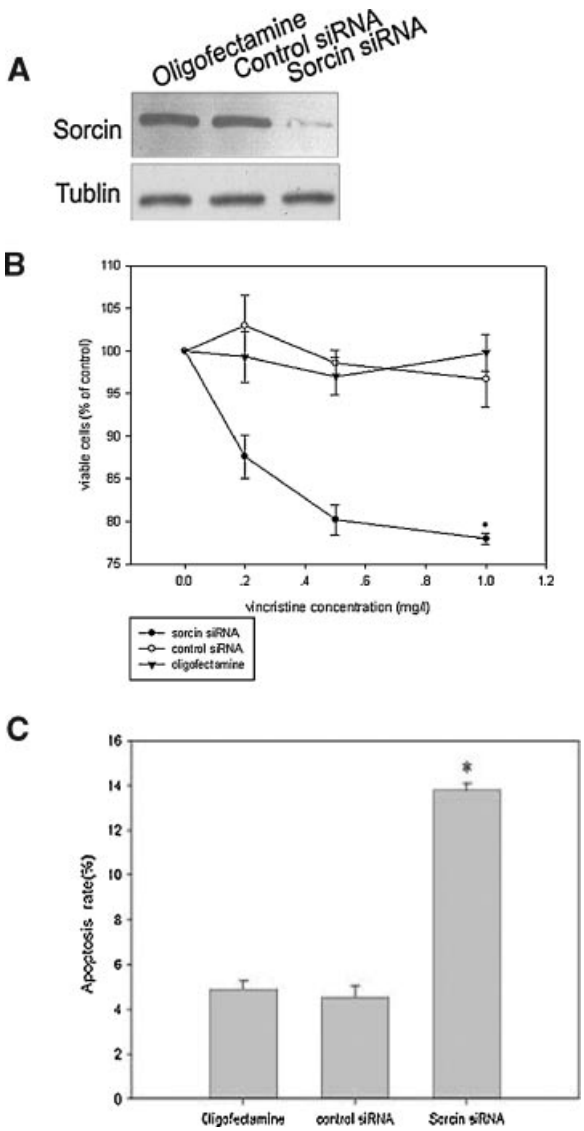


Fig. 3. A representative result showing changes in the expression levels of Annexin1, GRP78, EEF2, and Sorcin in SGC7901 and SGC7901/VCR by Western blot analysis (**A**) and 2-DE (**B**).



MDR cells with sorcin-amplification, its level of expression was not necessarily correlated directly with the degree of drug resistance, which suggested that sorcin overexpression in MDR cells is unlikely a phenomenon secondarily to amplification of mdr1 gene, and sorcin protein might have played an independent role in mediating the resistance of MDR cells to vincristine [Wang et al., 1995; Lee, 1996]. In this study, we found that when sorcin overexpression in SGC7901/VCR was suppressed by Sorcin siRNA, the cells increased the sensitivity to vincristine. Furthermore, transfection of full-length Sorcin gene to SGC7901 cells can cause resistance to vincristine. It was obvious that the overexpression of sorcin was involved in the MDR phenotype of SGC7901/VCR possibly by inhibiting vincristine-induced cell apoptosis. These results are in good consistent with those reported by Parekh et al. [2002] who transfected

Fig. 4. The effect of sorcin siRNA treatment on vincristine chemosensitivity in SGC7901/VCR cells. **A**: Western blot analysis showed that treatment of SGC7901/VCR cells with sorcin siRNA significantly reduced Sorcin protein levels (about 85%), whereas sorcin protein expressions was not significantly suppressed by control siRNA. **B**: MTT assay showed that sorcin siRNA transfection could significantly decrease the cell viability of SGC7901/VCR cells incubated with vincristine. The experiment was repeated in triplicate. Points, mean viable cells (% of control) from three experiments; bars, SE. $*P \leq 0.05$ differ from control siRNA and oligofectamine control by *t*-test. Oligofectamine, cells treated with oligofectmine only. **C**: Flow cytometric analysis showed that the fraction of cells undergoing apoptosis was significantly higher in SGC7901/VCR cells transfected with Sorcin siRNA than with control siRNA (14.5% vs. 4.2%). $*P \leq 0.05$ differ from control siRNA and oligofectamine control by *t*-test.

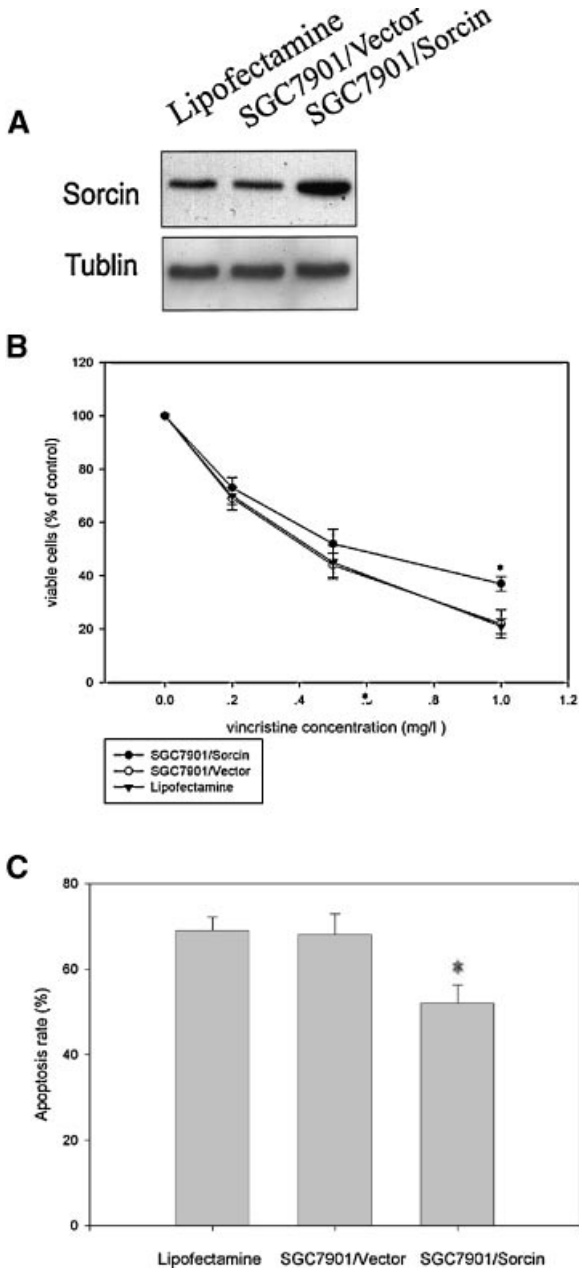


Fig. 5. The effect of enforced expression of sorcin on vincristine resistance in SGC7901 cells. **A**: After 48 h transfection, Western blot analysis showed that transfection of SGC7901 cells with pcDNA3.0/Sorcin significantly increased Sorcin protein levels (about 68%), compared with the control pcDNA3.0/Vector. **B**: MTT assay showed that pcDNA3.0/Sorcin transfection could cause resistance to vincristine. The experiment was repeated in triplicate. Points, mean viable cells (% of control) from three experiments; bars, SE. $*P \leq 0.05$ differ from control pcDNA3.0/Vector and lipofectamine control by *t*-test. Lipofectamine, cells treated with lipofectamine only. **C**: Flow cytometric analysis showed that the fraction of cells undergoing apoptosis was lower in SGC7901 cells transfected with pcDNA3.0/Sorcin than with pcDNA3.0/Vector (52.5% vs. 68.2%). $*P \leq 0.05$ differ from control pcDNA3.0/Vector and lipofectamine control by *t*-test.

human breast cancer cell with full-length cDNA of sorcin, and induced the low level resistance to paclitaxel in the transfectants. Reticulocalbin-1 (RCN) is an endoplasmic reticulum-resident calcium-binding protein with six repeats of a domain containing the "EF-hand" motif. Hirano et al. [2005] used the 2-DE analysis showing that decreased expression of RCN was associated with the acquisition of CDDP-drug resistance, and RCN-transfectant of lung cancer H69 CDDP-resistant strain showed intermediate sensitivity between the parent strain and the CDDP-resistant strain. In our case, compared with SGC7901, SGC7901/VCR had an obvious down-regulation of RCN also, which supported that the decreased expression of RCN is correlated with the MDR of cancer cells. SGC7901/VCR cells have markedly differential expression of annexin isoforms when compared with SGC7901. Annexin 5 was decreased, whereas annexin 1 was highly expressed in SGC7901/VCR. The annexins are a calcium-binding protein family and their function is not well understood. Although overexpression of annexin 1 in a gastric carcinoma cell line resistant to daunorubicin was reported by Sinha et al. [1998]. However, a rational correlation of annexin 1 with MDR remains unknown.

One of the major differentially expressed proteins groups is chaperones including members of heat shock proteins family, such as HSP27, HSC70, HSPA8 and GRP78, expression level changes of which were obvious in SGC7901/VCR as compared with SGC7901. HSPs comprise several different families of proteins that are induced in response to a wide variety of physiologic and environmental insults, and enable the cell to survive and recover from stressful conditions. Among them, HSP27 is obviously involved in the MDR phenotype by increasing level of glutathione (GSH) [Mehlen et al., 1997], decreasing the level of reactive oxygen species (ROS), stabilizing microfilaments and providing protection against toxicants [Lavoie et al., 1995]. Glucose-regulated protein 78 (GRP78) is associated with the MDR phenotype of many types of cancer cells. GRP78 can protect cells against apoptotic death by preventing the formation of a functional apoptosome [Reddy et al., 1999], and also complexes with caspase-7 to form an antiapoptotic complex [Reddy et al., 2003]. In our work, SGC7901/VCR had an obvious up-regulation of HSP27 and down-regulation of GRP78 as compared with

SGC7901, which supported that differential expression of HSP27 and GRP78 is correlated with the MDR of SGC7901/VCR cells.

The expression levels of some metabolic enzymes had obvious changes in SGC7901/VCR. L-lactate dehydrogenase B subunit (LDHB), also known as LDH-H, was found to be down-regulated in SGC7901/VCR as compared with SGC7901. This protein is a key enzyme in anaerobic cellular metabolism, and catalyzes the transformation of pyruvate to lactate. Although the detailed physiological functions of LDHB are still unknown, a few studies reported that LDHB expression changed in malignant tumors, such as that loss of LDHB expression in highly metastatic human prostate cancer cell line LNCaP-LN3 as compared to the low metastatic LNCaP cell line [Leiblich et al., 2006]. We have also observed the down-regulation of dUTP pyrophosphatase in SGC7901/VCR. dUTPase is a ubiquitous enzyme that catalyzes the hydrolysis of dUTP to dUMP and pyrophosphatase. dUTPase mediates a protective role by limiting the accumulation of dUTP pools and countering the cytotoxic effect of uracil misincorporation into DNA [Ladner et al., 2000]. Most of the identified metabolic enzymes, such as Creatine kinase B-type, inorganic pyrophosphatase, and 3-ketoacid-coenzyme A transferase 1, etc., have not known direct roles in drug resistance. However, these enzymes might mediate energy generation that is important for cells to survive chronic environmental stresses, such as drug attack. The correlation between these enzymes and MDR in SGC7901/VCR needs to further study.

Nucleophosmin, one of the major nucleolar phosphoproteins, which may function in the assembly and transport of ribosome, was observed to be overexpressed in SGC7901/VCR. Wu et al. [2002] demonstrated that nucleophosmin mRNA was increased after UV irradiation in parental HeLa cells. Transfection of HeLa cells with nucleophosmin antisense cDNA increased cellular sensitivity to UV-induced cell killing, which indicated that nucleophosmin overexpression may be correlated with the development of MDR in SGC7901/VCR. The expressions of proteins related to signal transduction, such as N-myc downstream-regulated gene 1 (NDRG1) and chloride intracellular channel protein1 (CLIC1), changed in SGC7901/VCR cell line. NDRG1 may play a regulatory role in differentiation and maturation of various types of cells. Overexpression of NDRG1 in carcinoma cell lines reduces their growth and induces their morphological changes [Guan et al., 2000]. CLIC1 is one of the very limited number of cloned nuclear ion channels, and its role in gastric cancer remains unclear. Recently, CLIC1 and intracellular chloride channels have been implicated in modifying cell cycle, apoptosis, cell adhesion, and cell motility [Valenzuela et al., 2000]. In this case, down-regulation of NDRG1 and CLIC1 expression in SGC7901/VCR might be correlated with the development of its MDR phenotype based on their functions. Finally, there were still some proteins including proteins relative to transcription and translation, such as elongation factor 2, which were found to be differentially expressed between SGC7901/VCR and SGC7901, have not been linked to MDR previously.

Resistance to anti-cancer drugs is one of major problems faced during chemotherapy of gastric cancer, but the mechanism of its MDR is still unclear. In this study, we focused our attention on those proteins that changed in expression levels in SGC7901/VCR, 30 differential expressional proteins possibly associated with MDR of SGC7901/VCR were identified by 2-DE in combination with MS using subfractionation proteins, and association of sorcin, one of the highly expressed proteins in SGC7901/VCR, with MDR was verified. These data were valuable for further to study the mechanism of MDR in human gastric cancer, and also provide some new clues for investigating other tumors MDR.

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