ORIGINAL PAPER

Identification of proteins differentially expressed in adriamycin-resistant (pumc-91/ADM) and parental (pumc-91) human bladder cancer cell lines by proteome analysis

Qian Meng · Ting Lei · Min Zhang · Jin Zhao · Xu-Hong Zhao · Man Zhang

Abstract

Purpose Resistance to chemotherapy drugs remains a difficult problem in bladder cancer treatment. Protein expression is an important factor underlying multidrug resistance (MDR) in bladder cancer. The aim of the study was to explore differentially expressed proteins responsible for MDR between an adriamycin-resistant human bladder cancer cell line (pumc-91/ADM) and its parental cell line (pumc-91).

Methods Two-dimensional gel electrophoresis (2-DE) combining image analysis was used to screen the differentially expressed protein spots between the pumc-91/ADM and pumc-91 cell lines. Then, the protein spots were identified using MALDI-TOF/TOF mass spectrometry. Among the identified proteins, annexin A2 (ANXA2) and nucleophosmin (NPM1) were then further verified using RT-PCR and Western blot analysis.

Results A total of 30 proteins, including 19 up-regulated and 11 down-regulated proteins, were successfully identified in pumc-91/ADM. According to their different functions, these 30 proteins were classified into 12 categories. Annexin A2 (ANXA2) and nucleophosmin (NPM1) were up-regulated in pumc-91/ADM compared with pumc-91.

Conclusion The proteins identified may have an important clinical significance in MDR, and ANXA2 and NPM1 may take part in mechanism of MDR in bladder cancer.

Keywords Bladder cancer · Multidrug resistance · Annexin A2 · Nucleophosmin

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Introduction

Bladder cancer is one of the most common malignancies worldwide. It is the second most common cancer affecting the genitourinary tract and is also the second most common genitourinary tract cancer lead to mortality (Ichimi et al. 2009). The biological characteristics of bladder cancer render it prone to drug resistance and recurrences. Despite the application of various therapies, approximately 35–45 % of bladder tumors will recur, and 10 % of patients will progress to muscle-invasive disease (Gingrich 2011). Chemotherapy is an important tool for bladder cancer treatment: systemic chemotherapy is effective for advanced tumors; intravesical chemotherapy is a primary therapy for superficial tumors and can help prevent recurrence. Adriamycin is an important drug used for systemic and intravesical chemotherapy against bladder cancer. However, owing to multidrug resistance (MDR), treatment with Adriamycin or other agents often fails. "MDR is a phenomenon whereby resistance to 1 anticancer drug is accompanied by resistance to drugs whose structures and mechanisms of action may be completely different (Baguley 2010)." Several mechanisms had been implicated in the development of MDR, such as "extrusion of the drug by cell membrane pumps, enhanced drug detoxification, increased DNA damage repair, redistribution of intracellular accumulation of drugs, modification of drug target molecules, suppression of drug-induced apoptosis, up-regulation of lipids" and other biochemical changes (Hong et al. 2005). Resistance to anticancer drugs remains a major obstacle to overcome, and further studies on MDR in bladder cancer are required. We have previously established a human bladder cancer cell line is resistant to adriamycin (pumc-91/ADM). In order to assess whether the pumc-91/ADM resistant to adriamycin, MTT and flow

cytometry analysis were performed. The results showed that pumc-91/ADM exhibited a tenfold resistance to adriamycin and were also cross-resistance to methotrexate, cisplatin, vincristine, and epirubicin. The pumc-91/ADM was a good model for further research (Zhang et al. 2009a, b).

In addition, protein expression is an important factor underlying MDR in bladder cancer. Two classical proteins related to MDR, P-glycoprotein (P-gp) and the multidrug resistance protein 1 (MRP1), were over-expressed in bladder cancer cells resistant to adriamycin and patients after chemotherapeutic treatment (Hasegawa et al. 1995; Nakagawa et al. 1997; Tada et al. 2000). Few other meaningful proteins associated with MDR were identified. In this study, we attempted to reveal new markers related to MDR in bladder cancer by examining protein expression changes between pumc-91/ADM and pumc-91.

Materials and methods

Reagents

Adriamycin (Sigma), IPG dry strips (pI 3–10, 24 cm), thiourea, urea, CHAPS, DTT, tris-base, SDS, TEMED, coomassie brilliant blue G-250, trizol, the enhanced HRP-DAB chromogenic kit. Horseradish-peroxidase-conjugated goat anti-mouse, goat anti-rabbit IgG (Santa Cruz), polyclonal antibodies against annexin2 (ANXA2, ab41803) and monoclonal antibodies against nucleophosmin (NPM1, ab10530) (Abcam).

Cell lines

The human bladder cancer cell line pumc-91 was provided from the Cell Laboratory of Beijing Union Medical College Hospital. When grew to the logarithmic phase, the pumc-91 cell was prepared into single cell suspension and incubated with adriamycin. We induced and established the multidrug-resistant bladder cancer cell lines pumc-91/ADM by increasing use of adriamycin. To preserve the biologic characteristics of MDR, pumc-91/ADM was cultured in RPMI1640 medium with 15 % fetal calf serum and 1.0 mg/L adriamycin.

Protein extraction

Pumc-91/ADM and pumc-91 cells were collected and washed three times with PBS, the cells were lysed in lysis buffer (Triscl 30 mM, thiourea 2 mol/L, urea 7 mol/L, 1 % DTT, 4 % CHAPS, 1 % Pharmalyte, 1 g/L DNase, pH adjusted to 8.5 with HCL) followed by sonication treatment (30 × 1 s, total 60 s) placed in a beaker with ice water mixture. The cell lysate was placed 30 min at room temperature and then centrifuged at 15,000 rpm for 30 min at 4 °C to obtain the supernatant. Protein concentrations were measured with the Bradford assay (Bradford 1976), and the extracted protein was packed and stored at −80 °C.

Two-dimensional gel electrophoresis (2-DE)

Two-dimensional gel electrophoresis (2-DE) was performed as described by the manufacturer's suggested protocols (Bio-Rad). The pumc-91/ADM and pumc-91 were run on 3 gels, respectively, to generate 6 2-DE maps for analysis. For each gel, the protein sample extracted was mixed with rehydration buffer (thiourea 2 mol/L, urea 7 mol/L, 1 % DTT, 4 % CHAPS, 1 % ampholyte and trace bromophenol blue), and the total volume was adjusted to 450 μL (1 mg of proteins). The IPG strip placed side down in a 24-cm focusing tray containing the protein sample solutions and then covered with mineral oil. Isoelectric focusing was carried out on PROTEAN IEF Cells (Bio-Rad). After rehydrating for 13 h at 50 V, the focusing was conducted successively according to the following conditions: 2 h at 250 V, 2 h at 500 V, 2 h at 1,000 V, and 5 h at 10,000 V to give a total of 120 kVh. First-dimension IPG strip was then equilibrated for 15 min in Equilibration Buffer I (6 M urea, 20 % glycerol, 2 % SDS, 0.375 M Tris–HCl pH 8.8, 0.1 g DTT) and transferred to the Equilibration Buffer II (6 M urea, 20 % glycerol, 2 % SDS, 0.375 M Tris–HCl pH 8.8, 0.4 g iodoacetamide) for 15 min. After equilibration, second-dimension sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 12 % polyacrylamide gels and used the PROTEAN-II XI Electrophoresis Cell (Bio-Rad) at 60 v/gel for 1 h and then at 160 v/gel for 20 h. Gels were then fixed in 10 % methanol and 7 % acetic acid for 1 h, stained with Coomassie blue G-250 (Bio-Rad) overnight followed by being destained with deionized water. Each stained 2-DE gels were scanned on Image scanner and analyzed using PDQuest software (Bio-Rad).

Image analysis

The scanned six 2-DE images (3 images of pumc-91/ADM, 3 images of pumc-91) were analyzed using PDQuest software (v.7.1.0). Protein spots of differential abundance were screened between the pumc-91/ADM and pumc-91 2-DE images. In order to reduce experimental variations, three separate gels were run on for each sample at the same time. The six images were normalized prior to analysis to eliminate the background interference. An automatic match-set of all 6 2-DE maps was conducted after a master gel had been constructed, followed by manually matched, and quantitative analysis of differential protein abundance through statistical analysis was performed. Abundance of

matched protein spots of the two images (pumc-91/ADM image and pumc-91 image) was defined as differential if the observed fold change was greater than 2 with p values less than 0.05.

Protein identification by MALDI-TOF/TOF

Differentially expressed protein spots confirmed by PDQuest software were excised from the gels, destained with 100 mmol/L NH_4HCO_3 in 50 % acetonitrile. After dried in a vacuum centrifuge, the gel particles were then digested with the 0.01 μg/μl trypsin and 25 mmol/L NH_4HCO_3 overnight at 37 °C. The next day, peptides were firstly extracted using extraction buffer I (5 % trifluoroacetic acid), followed by the second in the extraction buffer II (50 % acetonitrile, 2.5 % trifluoroacetic acid). The resulting peptides were subjected to the MS analysis dissolved in 7 mg/ml CHCC matrix in 50 % acetonitrile, 0.1 % trifluoroacetic acid.

MS and MS/MS spectra of the differentially expressed proteins were obtained by the ABI 4700 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems) with a mass range of 700–4,000 m/z. Before analysis, internal and external calibration was performed in MS and MS/MS mode. The peptides with signal-to-noise ratio above 100 at the MS were selected for the MS/MS. For MS/MS analysis, a minimum signal-to-noise ratio of 50 was set to be picked from each spot. The MS combining with MS/MS spectra was searched against the IPI human database using the MASCOT (Matrix Science) and GPS Explorer (Applied Biosystems). The searching parameters were set up as follows: homo sapiens, trypsin cleavage, the missed cleavage sites were allowed up to 1, carbamidomethylation was the fixed modification (cysteine); oxidation of methionine was the variable modification; the mass tolerance set to ±1.0 Dalton; the MS/MS tolerance set to ±0.5 Dalton.

Reverse transcription (RT)-PCR

Total RNA was extracted from the pumc-91/ADM and pumc-91 cells using the Trizol method according to manufacturer's protocol. Three micrograms of total RNA from each sample was reverse transcribed to obtain single-stranded cDNA. PCR assays were carried out in a PCR amplification instrument (Eppendorf). The gene anxa2, npm1, and β-actin were amplified using the specific primers (Table 1). The PCR conditions were 30–34 cycles, an annealing temperature of 55 °C was used for the anxa2 and npm1 primers, and 58 °C was used for the β-actin primers. All primers were annealed for 1 min and denatured at 94 °C for 40 s, elongated at 72 °C for 1 min. The PCR products were detected on a 1.5 % agarose gel with ethidium bromide staining. Using the Laned 1D gel

analysis software (Sage Creation, China), the integrated optical density (IOD) of each band was analyzed. Every experiment was repeated at least three times. To assess the differential mRNA level of anxa2 and npm1 between the two cell lines, the average IOD of the gen in the pumc-91/ADM was calculated relative to which in the pumc-91, after normalization against a β-actin standard. The Histogram for relative fold expression change of the gen was drawn using SPSS (v 16.0).

Western blotting

Western blotting was performed with proteins extracted from the two cell lines prepared for 2-DE. The process was done as previously described (Pastorelli et al. 2007). Briefly, total 30 μg proteins were separated by 10 % SDS-PAGE and transferred 1.5 h to a PVDF membrane (Bio-Rad). The membrane was blocked with 5 % nonfat dry milk for 2 h at room temperature and then incubated with primary annexin A2, nucleophosmin, and β-actin antibodies overnight at 4 °C. Horseradish-peroxidase-conjugated goat anti-mouse, goat anti-rabbit IgG were used as secondary antibodies. The results were detected by the enhanced HRP-DAB chromogenic kit.

Statistical analysis

The significant difference of proteins and relative mRNA expression levels of ANXA2, NPM1 between the pumc-91/ADM and pumc-91 cell lines was evaluated using Student's t-test. p values for the variance of protein and mRNA were determined with $p < 0.05$. Statistical analysis was done by using SPSS (v16.0).

Results

Identification of differentially expressed proteins

In order to compare global protein expression profiles between the drug-resistant cell line pumc-91/ADM and its parental cell line pumc-91, 2-DE analysis was performed. Protein spots of differential abundance between the 2 cell lines were screened through the PDQuest software for further analysis. The mean number of protein spots detected in pumc-91/ADM and pumc-91 2-DE maps was $1,772 \pm 57$ and $1,739 \pm 59$, respectively. Thirty-four protein spots with a more than twofold expressed change between the two cell lines (Fig. 1) were selected and submitted to MALDI-TOF/TOF for identification. A total of 30 proteins, including 19 up-regulated and 11 down-regulated proteins, differentially expressed in pumc-91/ADM (but not in pumc-91) were successfully identified.

Table 1 Primers for Reverse transcription (RT)-PCR

Genes	Forward (5′ → 3′)	Reverse (5′ → 3′)
ANXA2	5′-ATGTCTACTGTTCACGAAAT-3	5′-GCTCCTGGTTGGTTCTGG-3
β-actin	5′-CTACAATGAGCTGCGTGTGGC-3′	5′-CAGGTCCAGACGCAGGATGGC-3′
NPM1	5′-TGGTGCAAAGGATGAGTTGC-3′	5′-GTCATCATCTTCATCAGCAGC-3′
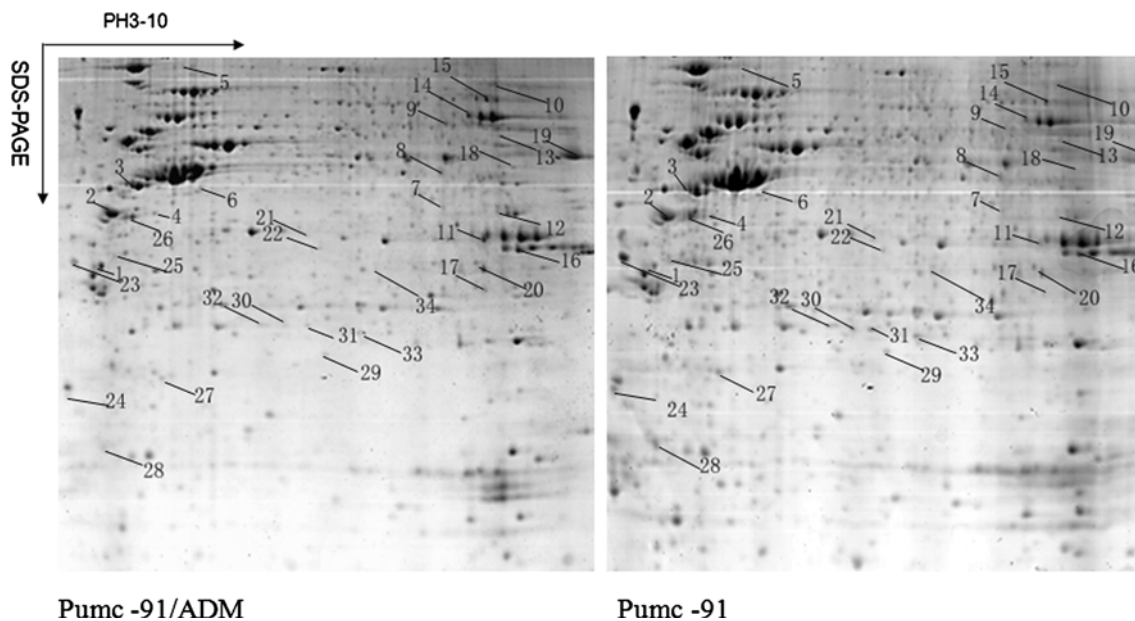
Fig. 1 Representative two-dimensional electrophoresis gels (pI 3–10, 24 cm) showed that 34 proteins of differential expression (signed the numbers) between the drug-resistant cell line pumc-91/ADM and its parental cell line pumc-91

The identification information of these proteins is shown in Table 2.

Of the 30 proteins, we choose ANXA2 and NPM1 for further verification by RT-PCR and Western blot. Peptide mass fingerprinting (PMF) and matched amino acid sequences of these proteins are illustrated in Fig. 2. Representative MS/MS spectra of ANXA2 (peptide sequence: AYTNFDAER) and NPM1 (peptide sequence: MSVQPTVSLGGFEITPP VVLR) are shown in Fig. 3, respectively.

The 30 proteins, which were differentially expressed between the pumc-91/ADM and pumc-91 cell lines, were further classified into 13 functional categories (Fig. 4). The categories were as follows: transferase (20 %), oxidoreductase (13.33 %), transporter (10 %), transcription factor (10 %), calcium ion binding (10 %), nucleic acid binding (6.67 %), enzyme modulator (6.67 %), cytoskeletal protein (6.67 %), signaling molecule (3.33 %), ligase (3.33 %), hydrolase (3.33 %), lyase (3.33 %), and storage protein (3.33 %).

Validation of differentially expressed proteins

To further verify the identified proteins, semi-quantitative RT-PCR analysis was performed to determine the mRNA

level of anxa2 and npm1 in the pumc-91/ADM and pumc-91 cell lines. Figure 5a, c showed the PCR products detected by agarose electrophoresis; the relative mRNA expression levels of the gens were up-regulated in pumc-91/ADM compared to the pumc-91 cell lines after normalization to a β-actin standard, and this finding was consistent with the results obtained by 2-DE. The histograms for relative mRNA fold expression change are shown in Fig. 5b, d.

Western blot analysis was also performed to determine the protein expression level of ANXA2 and NPM1 in the pumc-91/ADM and pumc-91 cell lines. As seen in Fig. 6, the relative expression levels of the proteins were up-regulated in the pumc-91/ADM compared to the pumc-91 cell line.

Discussion

Differential protein expression between the adriamycin-resistant human bladder cancer cell line pumc-91/ADM and its parental cell line pumc-91 was assessed using a classical 2-DE method and MALDI-TOF/TOF. A total of 30 proteins differentially expressed were discovered. Two

Table 2 Proteins identified by MALDI-TOF/TOF, showing 30 different expression proteins between the drug-resistant cell line pumc-91/ADM and its parental cell line pumc-91 (ratio: pumc-91/ADM vs pumc-91)

Spot no	SWISS-PROT	Protein name	Score	Mass (Da)	PI	Ratio	Function
1	P67936	Tropomyosin alpha-4 chain (TPM4)	168	28,504	4.67	2.15	Cytoskeleton
2	P06748	Nucleophosmin (NPM1)	103	32,554	4.64	2.63	Transporter
3	P14923	Junction-plakoglobin (JUP)	177	62,576	5.09	5.8	Storage
4	P05198	Eukaryotic translation initiation factor 2 subunit 1 (EIF2S1)	129	36,089	5.02	2.32	Nucleic acid binding
5	P04264	Keratin, type II cytoskeletal 1 (KRT1)	80	65,999	8.15	2.38	Cytoskeleton
6	O95433	Activator of 90 kDa heat shock-protein TPase homolog 1 (AHSA1)	104	38,250	5.41	2.83	Enzyme modulator
7	Q6N043	Zinc-finger protein 280D	65	34,315	8.68	3.26	Transcription
8	P22234	Multifunctional protein ADE2 (PAICS)	81	45,622	6.29	2.25	Ligase
9	P47895	Aldehyde dehydrogenase family 1 member A3 (ALDH1A3)	76	45,406	8.65	2.63	Oxidoreductase
10	P13639	Elongation factor 2 (EEF2)	150	95,277	6.41	3.8	Nucleotidyl transferase
11	P07355	Annexin A2 (ANXA2)	229	38,579	7.57	3.5	Calcium ion binding
12	P04075	Fructose-bisphosphate aldolase A (ALDOA)	61	39,395	8.3	3.11	Lyase
13	P34897	Serine hydroxymethyl-transferase mitochondrial (SHMT2)	97	53,421	8.35	2.38	Transferase
14	P14618	Pyruvate kinase isozymes M1/M2 (PKM2)	177	57,900	7.96	2.32	Transferase
15	P29401	Transketolase (TKT)	175	49,878	8.02	3.76	Transferase
16	P00338	L-lactate dehydrogenase A chain (LDHA)	137	36,665	8.44	2.5	Oxidoreductase
18	Q9NTK5	GTP-binding protein 9 (OLA1)	139	44,715	7.64	3.12	Enzyme modulator
19	P68104	Elongation factor 1-alpha 1 (EEF1A1)	69	50,109	9.1	4.34	Nucleotidyl transferase
20	P63244	Guanine nucleotide-binding protein subunit beta-2-like 1 (GNB2L1)	223	35,054	7.6	2.15	Signaling molecule
21	P04083	Annexin A1 (ANXA1)	230	38,690	6.57	−2.78	Calcium ion binding
22	P04083	Annexin A1 (ANXA1)	230	38,690	6.57	−2.78	Calcium ion binding
23	Q13765	Nascent polypeptide-associated complex subunit alpha (NACA)	198	23,369	4.52	−3.34	Transcription
25	Q9UKK9	ADP-sugar pyrophosphatase (NUDT5)	64	24,312	4.87	−3.83	Hydrolase
26	P07910	Heterogeneous nuclear-ribonucleoproteins C1/C2 (HNRNPC)	224	32,317	4.94	−2.11	Nucleic acid binding
27	A0PJH2	ATP5H protein	80	18,479	5.21	−2.21	Transporter
29	Q99497	Protein d J-1 (PARK7)	73	19,878	6.33	−4.43	Transcription
30	P30048	Thioredoxin-dependent peroxide reductase mitochondrial (PRDX3)	62	25,822	7.04	−4.45	Oxidoreductase
31	P30048	Thioredoxin-dependent peroxide reductase mitochondrial (PRDX3)	62	25,822	7.04	−4.45	Oxidoreductase
33	P30040	Endoplasmic reticulum resident protein 29 (ERP29)	123	28,975	6.77	−4.75	Transporter
34	Q13011	Delta(3,5)-delta(2,4)-dienoyl-CoA isomerase, mitochondrial (ECH1)	66	35,793	8.61	−3.55	Transferase

of these proteins—ANXA2 and NPM1—that were further verified by semi-quantitative RT-PCR, and Western blot analysis was over-expressed in the pumc-91/ADM cell line. These results suggested that various drug-resistant mechanisms may work together to induce chemotherapy resistance in bladder cancer.

ANXA2 is a 36-kDa protein belonging to the annexin superfamily of Ca^{2+}-dependent phospholipid-binding proteins. More than 60 different annexins have been identified, all of which play important roles in membrane traffic and organization, signal transduction, cell proliferation, differentiation, and apoptosis. More than ten annexin isoforms are expressed in human (Flood and Hajjar 2011; Hayes and Moss 2004; Gerke and Moss 2002). ANXA2 is also involved in angiogenesis, positive regulation of vesicle fusion, fibrinolysis, and other biological processes. It can

regulate cell proliferation and is over-expressed in many tumors, such as pancreatic (Vishwanatha et al. 1993), gastric (Emoto et al. 2001a, b), colorectal (Emoto et al. 2001a, b), and breast cancer (Sharma et al. 2006). ANXA2 can promote cell growth and thus may contribute to drug resistance. Studies had found that ANXA2 was up-regulated in some chemotherapeutic drug-resistance cell lines (Zhang et al. 2009a, b; Cole et al. 1992), which was in concordance with the results of the current study. Zhang

et al. (2009a, b) discovered that down-regulation of ANXA2 decreases cell proliferation in a drug-resistant MCF-7/ADM cell line. However, in prostate cancer, it had been reported that reduced or lost ANXA2 may lead to the development and progression of prostate cancer, and ANXA2 expression had been indicated to suppress cell migration (Liu et al. 2003). It is therefore apparent that the role of ANXA2 in different tumor types may rely on tumor origin and tissue specificity (Yao et al. 2009). We should
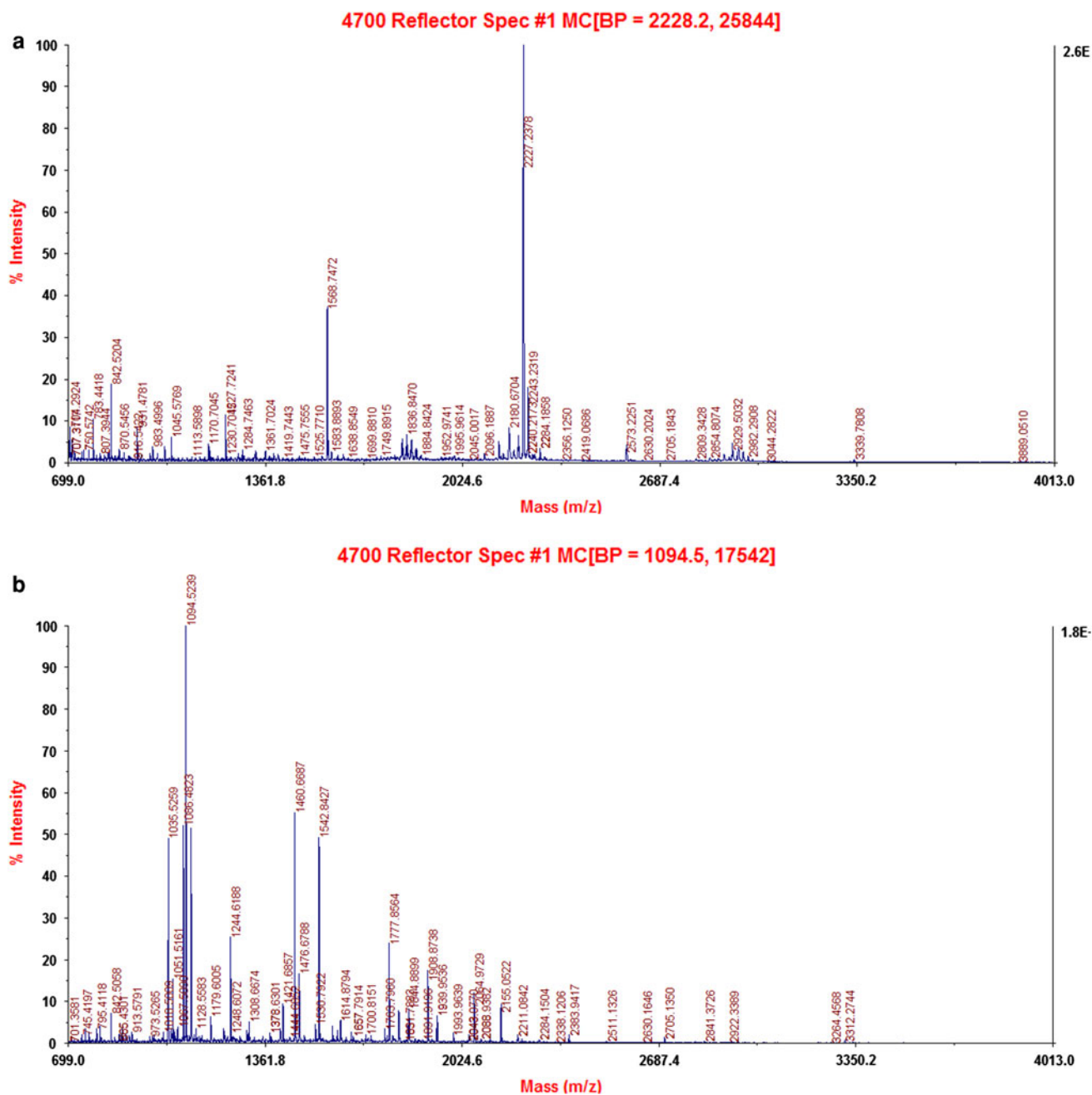

Fig. 2 Through MALDI-TOF/TOF analysis, the protein spots 11 and 2 were identified as ANXA2 and NPM1. The peptide mass fingerprinting (PMF) of ANXA2 and NPM1 is showed in (**a**, **b**). The matched amino acid sequences (marked with *red*) of ANXA2 and NPM1 are showed in (**c**, **d**)

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c
  1 MSTVHEILCK LSLEGDHSTP PSAYGSVKAY TNFDAERDAL NIETAIKTKG
 51 VDEVTIVNIL TNRSNAQRQD IAFAYQRRTK KELASALKSA LSGHLETVIL
101 GLLKTPAQYD ASELKASMKG LGTDEDSLIE IICSRTNQEL QEINRVYKEM
151 YKTDLEKDII SDTSGDFRKL MVALAKGRRA EDGSVIDYEL IDQDARDLYD
201 AGVKRKGTDV PKWISIMTER SVPHLQKVFD RYKSYSPYDM LESIRKEVKG
251 DLENAFLNLV QCIQNKPLYF ADRLYDSMKG KGTRDKVLIR IMVSRSEVDM
301 LKIRSEFKRK YGKSLYYYIQ QDTKGDYQKA LLYLCGGDD

d
  1 MEDSMDMDMS PLRPQNYLFG CELKADKDYH FKVDNDENEH QLSLRTVSLG
 51 AGAKDELHIV EAEAMNYEGS PIKVTLATLK MSVQPTVSLG GFEITPPVVL
101 RLKCGSGPVH ISGQHLVAVE EDAESEDEEE EDVKLLSISG KRSAPGGGSK
151 VPQKKVKLAA DEDDDDDDEE DDDEDDDDDD FDDEEAEEKA PVKKSIRDTP
201 AKNAQKSNQN GKDSKPSSTP RSKGQESFKK QEKTPKTPKG PSSVEDIKAK
251 MQASIEKGGS LPKVEAKFIN YVKNCFRMTD QEAIQDLWQW RKSL
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Fig. 2 continued

study the function of ANXA2 and its relationship with MDR in different tumors, respectively.

ANXA2 is also associated with metastasis and prognosis. It is a receptor for plasminogen and tissue-type plasminogen activator (tPA). When ANXA2 binds to plasminogen, the latter gets converted to plasmin. The plasmin can facilitate the degradation of the extracellular matrix to facilitate cell invasion and migration (Schulz et al. 2009). Sharma et al. reported that ANXA2 was expressed in invasive/metastatic MDA-MB231 cells but not in noninvasive/non-metastatic MCF-7 cells (Sharma et al. 2006). ANXA2 had been suggested to promote the invasion and metastasis of cancer cells. Moreover, drug resistance was possibly associated with cancer invasion and metastasis. Certain tumor cells selected for drug resistance were found to be more invasive and metastatic compared to nonresistant parental cells, and in some cases, tumors with increased metastatic potential were found to be more resistant to drugs than their primary counterparts (Liang et al. 2002). It is reasonable that ANXA2 may contribute to MDR by helping cancer cells invade and migrate. Zhang et al. also found that ANXA2 may enhance cell invasion of the drug-resistant MCF-7/ADM cells line (Zhang et al. 2009a, b). In summary, the over-expression of ANXA2 in the bladder cancer pumc-91/ADM cell line may promote cell growth, enhanced cell invasion and metastasis; thus, it may be a factor in drug resistance.

Nucleophosmin (NPM 1), also known as B23, is a 37-kDa/pI 5.1 protein (Yung et al. 1985). It is originally identified as a non-ribosomal nucleolar phosphoprotein found at high levels in the granular regions of the nucleolus (Lim and Wang 2006). The protein is multifunctional and involved in cell proliferation and differentiation (Liu et al. 2007), nucleosome assembly (Herrera et al. 1995), P53 regulation (Colombo et al. 2002), transportation (Borer et al. 1989), centrosome duplication (Tokuyama et al. 2001), and many other biological processes. NPM1 was highly up-regulated in proliferative cells, and it promoted cell growth by inhibiting tumor suppressors (Ye 2005). NPM1 was over-expressed in many tumors and had been considered a marker for colon (Nozawa et al. 1996), gastric (Tanaka et al. 1992), and prostate carcinomas (Subong et al. 1999) and thyroid tumors (Pianta et al. 2010). Additionally, NPM1 had been found to be mutated in acute myeloid leukemia (AML; Falini et al. 2005; Verhaak et al. 2005). Over-expression of NPM1 may be associated with tumor recurrence and progression and indicated a poor prognosis in bladder cancer and Ewing's sarcoma (Tsui et al. 2004, 2008; Kikuta et al. 2009). There are multiple mechanisms associated with the anti-apoptotic process of NPM1. It can interact with several transcription factors to promote cell growth, such as NF-κB, E2F1, pRB (Dhar et al. 2004; Lin et al. 2006), and p53 (Colombo et al. 2002). NPM1 is a factor in the ARF-p53 pathway, and it can compete with Mdm2 to bind with the tumor suppressor ARF and inhibit its function. Mutation and/or ectopic localization of the protein may be a key step in malignant transformation (Lim and Wang 2006; Korgaonkar et al. 2005).

NPM1 over-expression also prevents apoptosis induced by various pathways. For example, over-expression of NPM1 could not only reduce the susceptibility of human leukemia HL-60 cells to retinoic acid-induced apoptosis and differentiation (Hsu and Yung 2000), but it could also enhance the tolerance of NIH 3T3 cells and HeLa cells to ultraviolet radiation-induced cell death (Wu et al. 2002a, b). Yang et al. also reported that NPM1 was over-expressed in the gastric cancer drug-resistant SGC7901/ADM cell line, and that the suppression of NPM1 expression in the cell line enhanced adriamycin chemosensitivity (Yang et al. 2007). Recently, Hu et al. found that NPM1 may be associated with drug resistance and may aid in the prognostic assessment of leukemia cases (Hu et al. 2011). These results indicate that the over-expression of NPM1 may
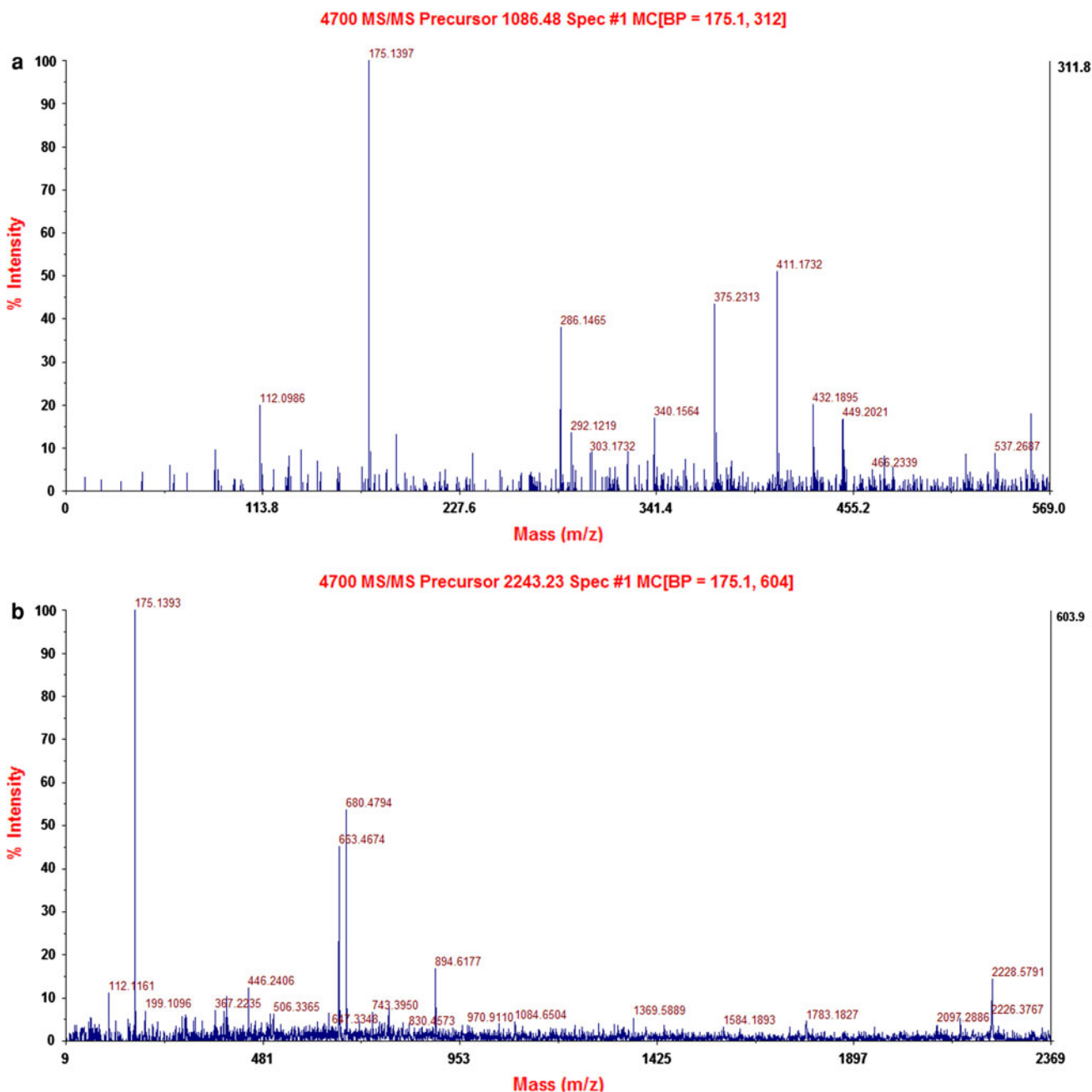
Fig. 3 A representative MS/MS spectrum of ANXA2 (m/z = 1,086.4807) and NPM1 (m/z = 2,243.2314), respectively, is showed in (**a**, **b**). The identified peptide sequence was AYTNFDAER and MSVQPTVSLGGFEITPPVVLR

Fig. 4 Pie chart showed the 13 functional categories as a percentage of the differentially expressed proteins
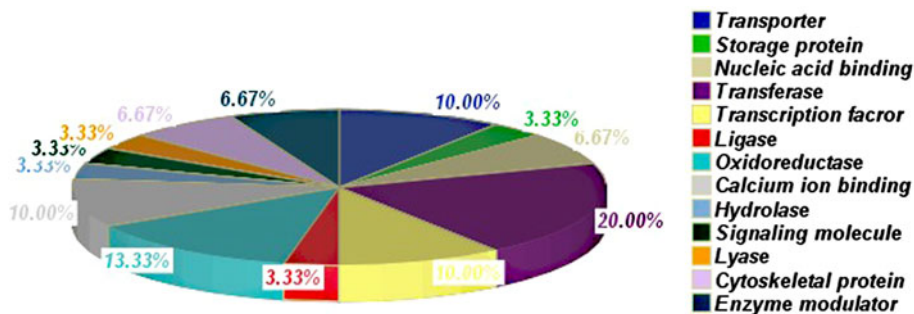
Fig. 5 a, **c** showed the results of ANXA2 and NPM1 PCR products. **b**, **d** The relative mRNA expression levels of them between the pumc-91/ADM and pumc-91 cell lines as verified by RT-PCR, after normalization against β-actin. RT-PCR analysis was repeated at least three times
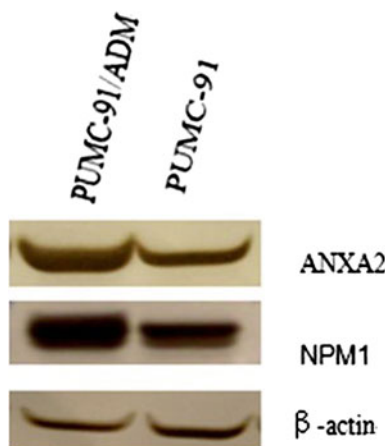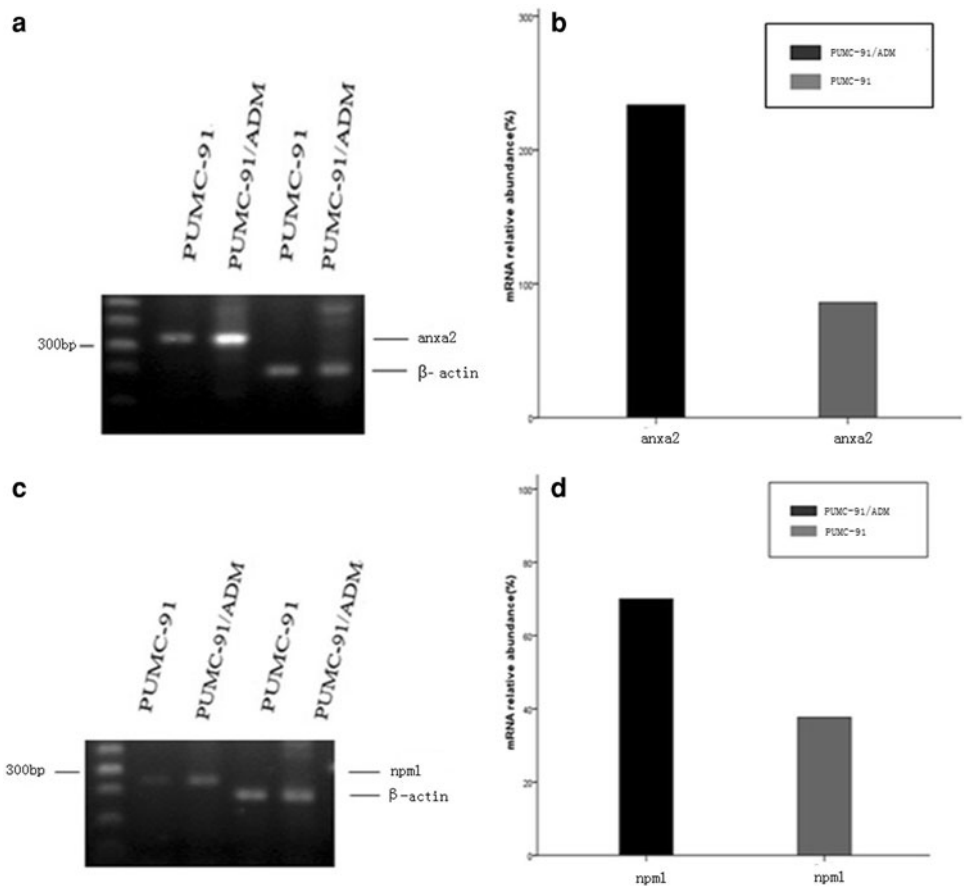

ANXA2 (Protein Spot 11) and NPM1 (Protein Spot 2) in the pumc-91/ADM and pumc-91 cell lines was verified by Western blot

Fig. 6 ANXA2 (Protein Spot 11) and NPM1 (Protein Spot 2) in the pumc-91/ADM and pumc-91 cell lines was verified by Western blot

have important implications in the bladder cancer pumc-91/ADM cell line. This protein possibly decreases adriamycin-induced cell apoptosis. In addition, as a chaperone protein, NPM1 can shuttle between the nucleus and cytoplasm, and thus, it may have some molecular chaperone activities such as "the promotion of the renaturation of chemically denatured proteins or the inhibition of

temperature-dependent and temperature-independent protein aggregation (Szebeni and Olson 1999)." Therefore, it is conceivable that NPM1 may play an important role in helping cells survive chemotherapy treatment.

In the 30 proteins differentially expressed identified in this study, 3 are involved in glycolysis: pyruvate kinase isozymes M1/M2 (PKM2), L-lactate dehydrogenase A chain (LDHA), and fructose-bisphosphate A (ALDOA). It is possible therefore that cancer cell glycolysis may have been enhanced by the addition of chemotherapeutic drugs in our experiment. The factors contributing to the enhanced glycolysis may be a result of mitochondrial metabolic defects that limit oxygen supply to tumor cells. The metabolic adaptation in response to these alterations is associated with reduced sensitivity to common anticancer agents (Xu et al. 2005). Xu et al. found that inhibiting glycolysis can induce apoptosis in multidrug-resistant cells (Xu et al. 2005), suggesting that inhibition of cellular glycolysis may be an effective way to overcome MDR. Subsequently, proteins involved in glycolysis may be new markers of MDR.

In addition to the previously described proteins, many other differentially expressed proteins with diverse functions, including transferase, oxidoreductase, transcription

factors, and signal transduction factors, were identified. Our results suggest that a variety of mechanisms and proteins may contribute to the development of MDR, although further verification and research are required to elucidate their exact roles.

Conclusion

Using a proteomic method, 30 differentially expressed proteins between pumc-91/ADM and pumc-91 cell lines were identified. Association of ANXA2 and NPM1, two of the upregulated proteins in pumc-91/ADM, were verified by RT-PCR and Western blot. These findings may have important clinical significance associate with MDR. Additionally, future studies would focus on the other identified proteins with similar strategies. All of the proteins in this study, including ANXA2 and NPM1, may be new targets for overcoming multidrug-resistant of bladder cancer.

Conflict of interest We declare that we have no conflict of interest.

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