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PAPER

Redox- and expression-proteomic analysis of plasma biomarkers in bladder transitional cell carcinoma†

Ying-Ray Lee,‡[ab] Yi-Wen Chen,‡[c] Ming-Chih Tsai,‡[c] Hsiu-Chuan Chou[d] and Hong-Lin Chan*[c]

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Recent progress in quantitative proteomics has offered opportunities in discovering plasma proteins as biomarkers to track the progression and understand the molecular mechanisms of bladder transitional cell carcinoma (TCC). In this study, differential plasma protein levels and redox regulation were analyzed by lysine- and cysteine-labeling two-dimensional differential gel electrophoresis (2D-DIGE), and combined with matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS/MS). This study shows 50 and 34 plasma protein features that were significantly changed in protein expression and thiol reactivity, respectively, and shows that plasma proteins involved in inflammatory responses are up-regulated in bladder TCC. In contrast, plasma proteins responsible for cytoskeleton and cytoskeleton regulation are down-regulated. In addition, plasma proteins involving cell adhesion, inflammatory responses, protease inhibitors, and plasma protein transport are shown to be altered in their thiol reactivity. In summary, we perform a comprehensive patient-based plasma proteomic approach for the identification of potential plasma biomarkers in bladder TCC screening and detection.

1. Introduction

Bladder cancer is one of the most common genitourinary cancers in the world. More than 90% of bladder tumors are diagnosed as bladder TCC.[1] The majority of bladder TCC are diagnosed as lower-grade superficial lesions and few lesions have been reported to develop into invasive tumors.[2] Thus, diagnosing bladder TCC at its early stage might significantly increase the cure rate and prevent its progression into invasive bladder cancer. Currently, the most reliable strategy for diagnosing bladder TCC is by cystoscopic examination through a bladder histological check.[3,4] However, the invasive nature of this procedure prevents it from conveniently detecting bladder TCC. Recent advances in bladder TCC diagnoses have introduced numerous noninvasive molecular diagnostic tools, such as proteomic analysis. To date, several urine-based bladder TCC markers have been identified by proteomic analysis including ADAM28,[5] nuclear matrix protein-22,[6] and fibrin/fibrinogen degradation products.[7] However, these are unlikely to replace cytology methods because of high false-positive rates and the low specificity for these identified markers.[8]

Inflammatory and oxidative reactions play important roles in pathogenesis including the progression of diabetes, cancer, and neurodegenerative diseases.[9–11] In the cancer research field, Yilmaz *et al.* reported that patients with bladder cancer were shown to have significantly lower levels of total plasma thiol groups in comparison to healthy donors.[9] Popadiuk *et al.* demonstrated that plasma protein carbonylation in childhood cancers were twice as high as in the healthy control group.[12] In addition, adult smokers and lung cancer patients have been reported with significantly high levels of plasma fibrinogen carbonylation.[13] In contrast, free thiol groups showed significantly lower levels in cancer patients, such as colorectal cancer in comparison with the healthy control groups.[14,15]

Two-dimensional gel electrophoresis (2DE) is one of the most commonly used proteomic strategies, which has often been used in profiling plasma proteins.[16,17] However, as most users realize, 2DE and other methods commonly used for in-gel protein analysis are inherently variable. Many replicate gels must be tested before significant differences in protein expression can be found with accuracy. Moreover, these protein visualisation methods often have narrow linear dynamic ranges of detection, rendering them inappropriate for the analysis of biological samples where protein copy numbers vary significantly. A significant improvement in the ability to use gel-based methods for protein

[a] *Department of Medical Research, Chiayi Chirsitan Hospital, Chiayi, Taiwan*
[b] *Min-Hwei College of Health Care Management, Tainan, Taiwan*
[c] *Institute of Bioinformatics and Structural Biology and Department of Medical Science, National Tsing Hua University, No. 101, Kuang-Fu Rd. Sec.2, Hsinchu, 30013, Taiwan. E-mail: hlchan@life.nthu.edu.tw; Fax: +886-3-5715934; Tel: +886-3-5742476*
[d] *Department of Applied Science, National Hsinchu University of Education, Hsinchu, Taiwan*
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‡ These authors contributed equally to this work.

quantitation and detection was achieved with the introduction of 2D-difference gel electrophoresis (2D-DIGE), which enables co-detection of several samples on the same 2-DE gel, thus avoiding gel-to-gel variation. This innovative technology relies on the pre-labeling of protein samples before electrophoresis with fluorescent dyes Cy2, Cy3, and Cy5, each exhibiting a distinct fluorescent wavelength to allow multiple experimental samples to include an internal standard. Thus, the samples can be concurrently separated in one gel. The internal standard, which is a pool of an equal amount of the experimental protein samples, can facilitate the data accuracy in normalization and increase statistical confidence in relative quantities across gels.[18–22] More recently, a cysteine labeling version of 2D-DIGE was developed, using ICy dyes (iodoacetyl cyanine dyes) which react with the free thiol group of cysteines through alkylation. The pairs of ICy dyes (ICy3 and ICy5) have been used to detect redox-dependent protein thiol modifications in a model cell system exposed to hydrogen peroxide, and in plasma fractions exposed to UVC.[23,24]

In this study, developing new techniques to detect bladder TCC is essential for both diagnosis and eventual prognosis. To examine the differentially-expressed levels and redox-regulation of plasma proteins associated with bladder TCC, a quantitative proteomics-based approach was performed using immunodepletion of high abundance proteins, cysteine- and lysine-2D-DIGE combining MALDI-TOF MS analysis to obtain a panel of plasma proteins found to be differentially altered between bladder TCC patients and healthy donors in protein abundance and thiol reactivity.

2. Materials and methods

2.1. Chemicals and reagents

Generic chemicals and an albumin and IgG depletion kit were purchased from Sigma-Aldrich (St. Louis, USA), and reagents for 2D-DIGE were purchased from GE Healthcare (Uppsala, Sweden). All the chemicals and biochemicals used in this study were of analytical grade. All primary antibodies were purchased from GeneTex (Hsinchu, Taiwan) and anti-mouse and anti-rabbit secondary antibodies were purchased from GE Healthcare (Uppsala, Sweden).

2.2. Plasma sample collection and purification

From January 2009 to December 2009, 20 donors with ages ranging from 50 to 60 y in a single hospital (Chiayi Christian Hospital, Chiayi, Taiwan) were enrolled in the study. Those included in the study were divided into bladder TCC patients (n = 15) and healthy donor groups (n = 5). The criteria to assess the presence of bladder TCC were based on the pathological diagnosis and guidelines proposed by the World Health Organization. Healthy individuals were selected with similar ages (50–60 y old) and with no bladder TCC clinically diagnosed. This study was approved by the Institutional Research Board and carried out according to the Helsinki Declaration Principles. Written informed consent was collected from all participating subjects.

Briefly, the whole blood with anticoagulant (EDTA) from the TCC patients and health donors were collected. The plasma was immediately separated, collected and stored at −80 °C without protease inhibitors. To improve the performance of the proteomic analysis of the plasma samples, the albumin and immunoglobulin G in the collected plasma samples were depleted using an albumin and IgG removal kit (Sigma, St. Louis, USA) in accordance with manufacturer instructions. The depleted plasma samples were precipitated by adding 1 volume of 100% TCA (at −20 °C) to 4 volumes of sample and incubated for 10 min at 4 °C. The precipitated protein was then recovered by centrifugation at 13 000 rpm for 10 min, and the resulting pellet was washed twice with ice-cold acetone. Air-dried pellets were resuspended in a 2-DE lysis buffer containing 4% w/v CHAPS, 7 M urea, 2 M thiourea, 10 mM Tris–HCl, pH 8.3, 1 mM EDTA for lysine-labeling 2D-DIGE analysis or they were resuspended in buffer containing 4% w/v CHAPS, 8 M urea, 10 mM Tris–HCl, pH 8.3, 1 mM EDTA for cysteine-labeling 2D-DIGE analysis.

2.3. Sample preparation for 2D-DIGE and gel image analysis

The plasma protein pellets were dissolved in 2-DE lysis buffer, and protein concentrations were determined using a Coomassie protein assay reagent (BioRad). Before performing 2D-DIGE, plasma samples from 15 bladder TCC patients and 5 healthy donors were pooled individually. After protein quantification with the Bradford assay, 100 μg of protein samples from patients and healthy donors were labeled with 250 pmol of either Cy3 or Cy5 for triplicate comparison on three 2-DE, respectively. To facilitate image matching and cross-gel statistical comparison, a pool of all samples was also prepared and labeled with Cy2 at a molar ratio of 2.5 pmol Cy2 per μg of protein as an internal standard for all gels. Thus, the triplicate samples and the internal standard could be run and quantified on multiple 2-DE. The detailed procedures for fluorescence dye-labeling, isoelectric focusing electrophoresis, 2DE, fluorescence image detection/analysis were described in our previous study.[19] Spots displaying in all 9 gel images (3 images per gel) with an $\approx$1.5 average-fold increase or decrease in abundance and a p-value of <0.05 were selected for protein identification.

For redox-DIGE analysis, protein samples were labeled with ICy3 or ICy5 (80 pmol μg^{-1} protein) on ice. Test samples were labeled with the ICy3 dye and mixed with an equal amount of a standard pool of both samples and labeled with ICy5. The reactions were left in the dark for 1 h followed by labeling with Cy2 for a further 30 min to facilitate subsequent image matching between dye-labeled proteins and colloidal Coomassie blue-stained proteins. The reactions were quenched with DTT (65 mM final concentration) for 10 min followed by L-lysine for a further 10 min. Volumes were adjusted to 450 μl with buffer plus DTT and IPG buffer for rehydration. Individual pooled samples were run in duplicate against the standard pool.[25]

2.4. Protein staining, in-gel digestion and MALDI-TOF MS analysis

Colloidal Coomassie blue G-250 staining was used to visualize CyDye-labeled protein features in 2-DE, followed by excised interested post-stained gel pieces for MALDI-TOF MS identification. The detailed procedures for protein staining, in-gel digestion, MALDI-TOF MS analysis, and the algorithm used for data processing have been described in our previous publication.[19]

The spectrometer was calibrated with a peptide calibration standard (Bruker Daltonics), and internal calibration was performed using trypsin autolysis peaks at m/z 842.51 and m/z 2211.10. Peaks in the mass range of m/z 800–3000 were used to generate a peptide mass fingerprint that was searched against the Swiss-Prot/TrEMBL database (released on December 2011) with 533 657 entries using Mascot software v2.3.02 (Matrix Science, London, UK). The following parameters were used for the search: *Homo sapiens*; tryptic digest with a maximum of 1 missed cleavage; carbamidomethylation of cysteine, partial protein N-terminal acetylation, partial methionine oxidation, and partial modification of glutamine to pyroglutamate, and a mass tolerance of 50 ppm. Identification was accepted based on significant MASCOT Mowse scores ($p < 0.05$), spectrum annotation, observed *versus* expected molecular weight, and p*I* on 2-DE.

2.5. Immunoblotting analysis and ELISA analysis

Immunoblotting and ELISA analysis were used to validate the differential abundance of mass spectrometry identified proteins. The detailed experimental procedures have been described in our previous study.[26–28] All primary antibodies used for expression validation were purchased from Genetex (Hsinchu, Taiwan).

3. Results

3.1. 2D-DIGE and mass spectrometry analysis of the immunodepleted plasma proteome

Because IgG and albumin account for approximately 70%–90% plasma proteins in humans, these high abundant proteins are obstacles for proteomic analysis. Accordingly, removing the high-abundance IgG and albumin from plasma samples can increase the viability of the low- and middle-abundance proteins, and enable accurate analysis. In this study, the high abundance proteins were removed before performing 2D-DIGE analysis.

To study the alteration of plasma protein in the bladder TCC patients, comparative proteomics analysis was performed between bladder TCC patients and healthy donors. The 2-DE images of the samples of the 2 groups were minimally labeled with Cy3 and Cy5 dyes and distributed to each gel. A pool of both samples was also prepared for labeling with Cy2 as an internal standard to run on all gels to facilitate image matching across gels. The plasma sample arrangement for a triplicate 2D-DIGE experiment is shown in Fig. 1A. Thus, the triplicate samples resolved in different gels can be quantitatively analyzed by means of the internal standard on multiple 2-DE. After resolving protein samples with the 2D-DIGE technique, the DeCyder image analysis software indicated that 101 protein features were showing a greater than 1.5-fold change in expression levels with student t-test (p-value) of less than 0.05. MALDI-TOF MS identification showed that 50 proteins (corresponding to 26 unique genes) were differentially expressed (Fig. 1B and C, ESI† Fig. S1, and Table 1). Representative examples for the evaluation by DeCyder of alteration in spot intensities using the 2D-DIGE system are shown in Fig. 2. To display visual alterations in corresponding spot intensity proportions, selected identified spots (carbonic anhydrase 1, serum amyloid protein, 14-3-3 protein, CD5 antigen-like protein, beta-2-glycoprotein, and haptoglobin) are shown as 3D images, and the

A

	Cy2	Cy3	Cy5
Gel1	Pool	Normal	TCC
Gel2	Pool	TCC	Normal
Gel3	Pool	Normal	TCC

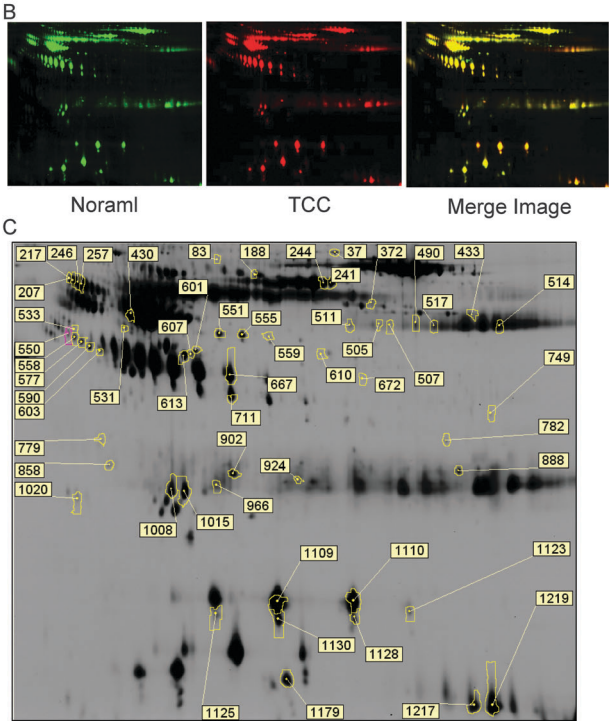

Fig. 1 2D-DIGE comparative analysis of plasma from bladder TCC cases and healthy controls. (A) Pooled plasma sample arrangement for a triplicate 2D-DIGE experiment. (B) Plasma samples (100 μg each) were labeled with Cy-dyes, and separated using 24 cm, pH 3–10 non-linear IPG strips. (C) The differentially expressed identified protein features in 2D-DIGE are annotated with spot numbers.

associated graph views of standardized abundances of the selected spots and spot locations (Fig. 2).

3.2. Validation by immunoblotting and ELISA

To verify the abundances of proteins deduced from the results of 2D-DIGE and MALDI-TOF MS/MS analysis, the abundance levels of identified carbonic anhydrase 1, haptoglobin, actin, and leucine-rich alpha-2-glycoprotein were investigated by immunoblotting and ELISA, respectively. As shown in Fig. 3, the 29 kDa carbonic anhydrase 1, the 38 kDa leucine-rich alpha-2-glycoprotein, and the 45 kDa haptoglobin were increased in the plasma of bladder TCC patients. In addition, the 42 kDa actin was significantly decreased in the plasma of patients with bladder TCC. These immunoblotting and ELISA results are consistent with the data from the 2D-DIGE and MALDI-TOF MS, and further suggest that they may be employed as potential signatures for the diagnosis of bladder TCC.

3.3. Redox 2D-DIGE analysis of differential cysteine-modification in bladder TCC plasma

Bladder TCC and numerous cancers have been reported to induce ROS generation and damage or deregulate cellular/plasma proteins through oxidative modification of their cysteinyl thiol groups.[29,30]

Table 1 List of differentially expressed plasma proteins between the bladder TCC patient (n = 15) and healthy donor groups (n = 5) as identified by MALDI-TOF/TOF MS following 2D-DIGE analysis

Spot No.	Swissprot No.	Protein name	Pred. M_W	Pred. PI	Cov. %	MASCOT score	No. of peptides matched/ supplied	t-Test	TCC/ normal[c]	Function	Peptide sequence
858	Q6NUR9	14-3-3 Protein zeta/delta	27 899	4.7	25	78/56	6/17	0.00036	−4.3	Signal transduction	FLIPNASQAESK DSTLIMQLLR
607	Q96HG5	Actin, cytoplasmic 1[a]	42 052	5.3	24	70/56	8/24	1.40×10^{-5}	−5.8	Cytoskeleton	GYSFTTTAER AVFPSIVGRPR
613	Q96HG5	Actin, cytoplasmic 1[a]	42 052	5.3	42	119/56	14/46	3.00×10^{-7}	−5.84	Cytoskeleton	IIAPPERK GYSFTTTAER
207	Q6NSC9	Alpha-1-antichymotrypsin[a]	47 792	5.3	15	63/56	6/16	0.00014	1.5	Inflammatory response	ITLLSALVETR WRDSLEFR
217	Q6NSC9	Alpha-1-antichymotrypsin[a]	47 792	5.3	25	114/56	10/20	1.20×10^{-5}	1.66	Inflammatory response	LINDYVK EIGELYLPK
246	Q6NSC9	Alpha-1-antichymotrypsin[a]	47 792	5.3	18	76/56	11/32	0.00023	1.64	Inflammatory response	WRDSLEFR ITLLSALVETR
257	Q6NSC9	Alpha-1-antichymotrypsin[a]	47 792	5.3	34	137/56	14/40	0.002	1.6	Inflammatory response	AKWEMPFDPQDTHQSR ITLLSALVETR
531	P01009	Alpha-1-antitrypsin	46 878	5.4	17	57/56	6/23	0.0012	1.51	Inflammatory response	LSITGTYDLK SVLGQLGITK
1008	P02647	Apolipoprotein A-I[a]	30 759	5.6	24	61/56	6/18	7.30×10^{-5}	−1.53	Inflammatory response	AKPALEDLR VQPYLDDFQK
1015	P02647	Apolipoprotein A-I[a]	30 759	5.6	53	138/56	17/54	3.90×10^{-5}	−1.62	Transport	AKPALEDLR ETEGLRQEMSK
372	P02749	Beta-2-glycoprotein 1	39 584	8.3	22	60/56	4/19	0.00037	−1.99	Blood coagulation	VCPFAGILENGAVR TFYEPGEEITYSCKPGYVSR
888	P00915	Carbonic anhydrase 1	28 909	6.6	38	57/56	6/37	0.0001	1.63	Transport	GGPFSDSYR YSAELHVAHWNSAK
601	O43866	CD5 antigen-like	39 603	5.3	37	120/56	11/27	2.30×10^{-5}	−1.71	Inflammatory response	LVGGDNLCSGR CSGEEQSLEQCQHR
782	Q5JQM8	Complement C4-A	2×10^{-5}	6.7	5	59/56	6/14	0.0000079	−1.81	Inflammatory response	GLQDEDGYR FACYYPR
749	P02671	Fibrinogen alpha chain	95 656	5.7	18	126/56	24/59	0.0011	1.59	Inflammatory response	GDFSSANNRDNTYNR MELERPGGNEITR
433	P02675	Fibrinogen beta chain[a]	56 577	8.5	36	162/56	22/40	0.0094	1.51	Blood coagulation	GGETSEMYLIQPDSSVKPYR GSWYSMR
505	P02675	Fibrinogen beta chain[a]	56 577	8.5	15	66/56	6/25	0.0014	2.28	Blood coagulation	IRPFFPQQ QDGSVDFGR
511	P02675	Fibrinogen beta chain[a]	56 577	8.5	23	82/56	9/28	0.0024	2.1	Blood coagulation	QDGSVDFGR EDGGGWWYNR
551	Q9UC63	Fibrinogen gamma chain[a]	52 106	5.4	48	144/56	18/61	2.70×10^{-5}	−1.73	Blood coagulation	QSGLYFIKPLK TRWYSMK
555	Q9UC63	Fibrinogen gamma chain[a]	52 106	5.4	37	131/56	12/29	7.00×10^{-5}	−1.51	Blood coagulation	VGPEADKYR DNCCILDER
559	Q9UC63	Fibrinogen gamma chain[a,b]	52 106	5.4	41 (3)	146/56 (75/31)	14/34 (1/1)	0.0057	−1.63	Blood coagulation	YLQEIYNSNNQK VGPEADKYR (IHLISTQSAIPYALR)
37	P06396	Gelsolin	86 043	5.9	18	63/56	13/52	0.042	−1.63	Cytoskeleton	HVVPNEVVVQR AGALNSNDAFVLK
667	P00738	Haptoglobin[a]	45 861	6.1	33	122/56	15/59	3.80×10^{-5}	1.5	Inflammatory response	LRTEGDGVYTLNNEK DIAPTLTLYVGKK
1109	P00738	Haptoglobin[a]	45 861	6.1	19	67/56	6/8	2.40×10^{-6}	2.05	Inflammatory response	KQWINK LRTEGDGVYTLNNEK
1110	P00738	Haptoglobin[a]	45 861	6.1	13	57/56	4/8	2.60×10^{-5}	1.82	Inflammatory response	NPANPVQR LRTEGDGVYTLNN
1123	P00738	Haptoglobin[a]	45 861	6.1	14	59/56	5/15	1.40×10^{-5}	1.92	Inflammatory response	AVGDKLPECEAVCGKPK TEGDGVYTLNDK
1125	P00738	Haptoglobin[a]	45 861	6.1	11	69/56	5/8	5.30×10^{-6}	2.03	Inflammatory response	TEGDGVYTLNNEK LRTEGDGVYTLNDK
1130	P00738	Haptoglobin[a]	45 861	6.1	8	59/56	5/12	0.00072	1.95	Inflammatory response	NYYKLR TEGDGVYTLNNEK

Table 1 (*continued*)

Spot No.	Swissprot No.	Protein name	Pred. M_W	Pred. P*I*	Cov. %	MASCOT score	No. of peptides matched/ supplied	*t*-Test	TCC/ normal[c]	Function	Peptide sequence
1179	P00738	Haptoglobin[a]	45 861	6.1	13	62/56	5/15	0.00014	−1.5	Inflammatory response	NPANPVQR AVGDKLPECEAVCGKPK
711	P00738	Haptoglobin[a,b]	45 861	6.1	16 (3)	57/56 (114/29)	7/12 (1/1)	1.30×10^{-5}	1.53	Inflammatory response	GSFPWQAK VGYVSGWGR (VVLHPNYSQVDIGLIK)
1128	P00738	Haptoglobin[a,b]	45 861	6.1	14 (3)	65/56 (42/29)	6/23 (1/1)	7.30×10^{-6}	1.75	Inflammatory response	NPANPVQR LRTEGDGVYTLNDK (IHLISTQSAIPYALR)
1217	P68871	Hemoglobin subunit beta[a]	16 102	6.8	38	84/56	5/10	4.10×10^{-5}	1.82	Transport	VVAGVANALAHKYH LLVVYPWTQ
1219	P68871	Hemoglobin subunit beta[a]	16 102	6.8	30	64/56	4/18	1.50×10^{-5}	2.12	Transport	FFESFGDLSTPDAVMGNPK LHVDPENFR
188	P02790	Hemopexin[a]	52 385	6.6	23	93/56	8/17	0.00033	−1.63	Transport	RLWWLDLK NFPSPVDAAFR
507	P02790	Hemopexin[a]	52 385	6.6	22	75/56	7/19	0.00011	2.28	Transport	DYFMPCPGR YYCFQGNQFLR
244	P01877	Ig alpha-2 chain C region	37 301	5.7	20	64/56	4/14	0.049	−1.58	Inflammatory response	WLQGSQELPR GDTFSCMVGHEALPLAFTQK
514	P01857	Ig gamma-1 chain C region	36 596	8.5	51	101/56	12/50	0.00025	1.59	Inflammatory response	FNWYVDGVEVHNAK STSGGTAALGCLVK
490	P01859	Ig gamma-2 chain C region[a]	36 505	7.7	28	56/56	5/20	1.60×10^{-6}	2.32	Inflammatory response	GPSVFPLAPCSR CCVECPPCPAPPV
517	P01859	Ig gamma-2 chain C region[a]	36 505	7.7	34	69/56	9/34	4.50×10^{-5}	2.38	Inflammatory response	NQVSLTCLVK GPSVFPLAPCSR
966	P01834	Ig kappa chain C region	11 773	5.6	48	57/56	3/17	0.0038	−1.52	Inflammatory response	AELQEGAR KWQEEMELYR
1020	P01591	Immunoglobulin J chain	18 543	5.1	28	57/56	4/11	7.30×10^{-5}	−1.53	Transport	FVYHLSDLCK IIVPLNNR
550	P02750	Leucine-rich alpha-2-glycoprotein[a]	38 382	6.5	20	57/56	5/15	5.60×10^{-5}	1.72	Inflammatory response	DLLLPQPDLR ALGHLDLSGNR
558	P02750	Leucine-rich alpha-2-glycoprotein[a]	38 382	6.5	17	59/56	4/15	8.60×10^{-7}	1.92	Inflammatory response	VAAGAFQGLR ALGHLDLSGNR
577	P02750	Leucine-rich alpha-2-glycoprotein[a]	38 382	6.5	27	70/56	6/18	3.10×10^{-5}	2.02	Inflammatory response	VAAGAFQGLR DLLLPQPDLR
603	P02750	Leucine-rich alpha-2-glycoprotein[a]	38 382	6.5	14	60/56	4/12	0.0017	1.62	Inflammatory response	ALGHLDLSGNR VAAGAFQGLR
590	P02750	Leucine-rich alpha-2-glycoprotein[a,b]	38 382	6.5	12 (2)	63/56 (61/31)	5/11 (1/1)	7.10×10^{-6}	1.91	Inflammatory response	VAAGAFQGLR ALGHLDLSGNR (VAAGAFQGLR)
83	P57772	Selenocysteine-specific elongation factor	65 890	8.6	11	76/56	6/8	0.0073	1.99	protein synthesis	DLTPAVTDNDEADKK AMDDYSVIGRSLFK
241	Q9P157	Serum albumin	71 317	5.9	27	153/56	14/22	0.001	−1.69	Transport	HPDYSVVLLLR YLYEIAR
902	P02743	Serum amyloid P-component	25 485	###	21	61/56	4/8	4.00×10^{-5}	1.84	Transport	GYVIIKPLVWV VGEYSLYIGR
779	Q9UCS3	Tropomyosin alpha-4 chain	28 619	4.7	19	89/56	7/11	0.0046	−3.55	Cytoskeleton	YEEVARK LVILEGELER

[a] Proteins appearing more than once were identified as isoforms with different percentage coverage of analyzed peptides, matched peptide numbers, MOWSE scores, average fold-differences, *t*-test values, and matched peptide sequences for each identified protein. [b] These proteins have been identified by both MALDI-TOF MS and MALDI-TOF/TOF MS. The parameters (sequence coverages, MASCOT values, matched peptide numbers, and matched peptide sequences) shown in brackets are obtained from MALDI-TOF/TOF MS sequence analysis. [c] Average fold-differences of replicate samples run on different gels from DeCyder analysis show abundance ratios for bladder TCC plasma *versus* healthy donor's plasma. Proteins displaying an average fold-difference of 1.5-fold up- or down-regulation where $p < 0.05$, and spots matched in all images are shown in the table. Functions were adopted from the Swiss-Prot database and research.

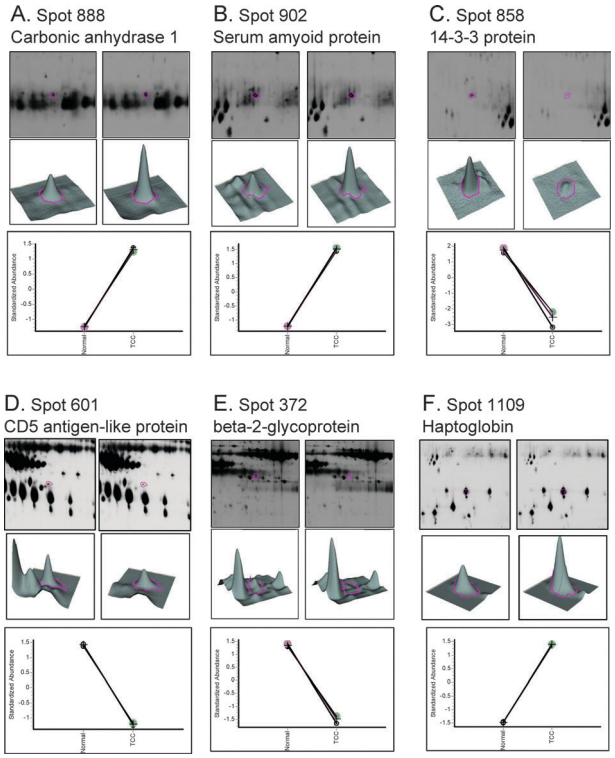

Fig. 2 Representative images of the identified spots ((A) carbonic anhydrase 1; (B) serum amyloid protein; (C) 14-3-3 protein; (D) CD5 antigen-like protein; (E) beta-2-glycoprotein; (F) haptoglobin) displaying bladder TCC-dependent protein expression changes. The levels of these proteins are visualized as 2-DE images (top panels), 3D spot images (middle panels), and protein expression maps (bottom panels).

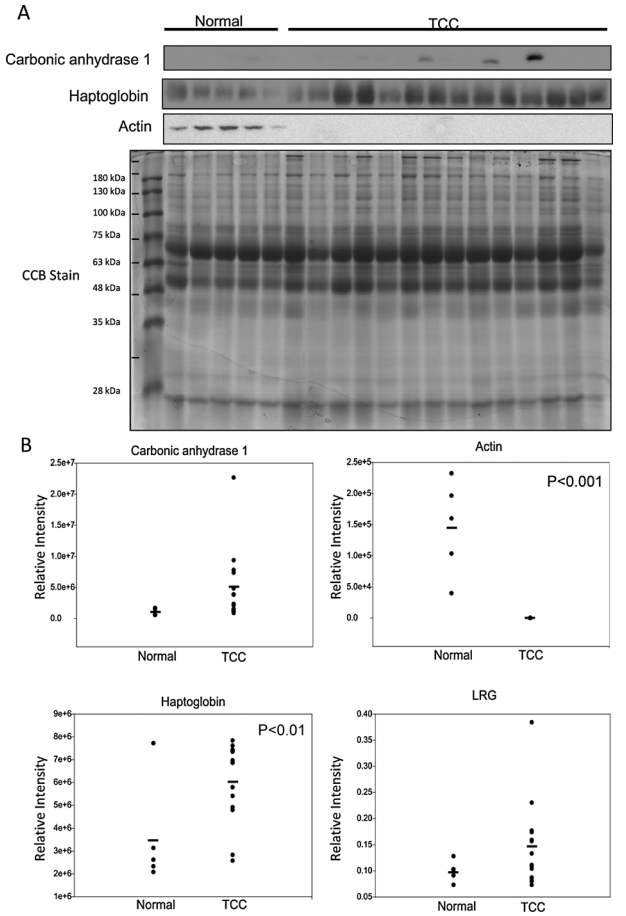

Fig. 3 Immunoblotting and ELISA analysis of carbonic anhydrase 1, haptoglobin, leucine-rich alpha-2-glycoprotein, and actin in bladder TCC plasma and healthy control plasma. (A) 20 μg of the albumin-depleted plasma were loaded and resolved by SDS-PAGE, followed by immunoblotting with carbonic anhydrase 1, haptoglobin and actin antibodies or by staining with colloidal Coomassie blue G-250 as a loading control. (B) Individual immunoblotting and ELISA histograms show relative intensities of carbonic anhydrase 1, haptoglobin, actin, and leucine-rich alpha-2-glycoprotein expression in normal controls and in healthy donors. The intensities of carbonic anhydrase 1, haptoglobin and actin were directly quantified by the band volume for each sample image by imageQuant software (GE healthcare). In contrast, the intensity of leucine-rich alpha-2-glycoprotein was obtained from ELISA analysis and absorbance was measured at 450 nm using a Stat Fax 2100 microtiter plate reader.

To investigate the plasma targets of these oxidative modifications in bladder TCC, we applied a recently developed redox 2D-DIGE methodology using iodoacetylated ICy dyes[31] to assess possible bladder TCC-induced changes in plasma protein thiol reactivity. Pooled plasma from bladder TCC and healthy donors were labeled with ICy3 in triplicate. Individual ICy3-labeled samples were then run on 2D gels against an equal load of ICy5-labeled standard pool composed of an equal mixture of both sample types (Fig. 4). After normalization with protein levels and analysis with DeCyder software, 174 protein features displayed significant differences with a cut-off of >1.5 average-fold increase or decrease in labeling (Fig. 4B). CCB post-staining and matching with fluorescence images permitted confident selection and identification of 34 proteins corresponding to 16 unique gene products by MALDI-TOF peptide mass fingerprinting and MALDI-TOF/TOF sequence analysis (Table 2 and ESI† Fig. 2). All of the identified proteins contain a minimum of one cysteine residue, and because the ICy dyes target reduced cysteinyl thiols, these results suggest an altered oxidative status of certain thiol groups in bladder TCC.

Representative examples for evaluation by DeCyder of alteration in protein thiol reactivity using the 2D-DIGE system are shown in Fig. 5. To display visual alterations in corresponding spot thiol reactivity proportions, selected identified spots (serine/threonine-protein kinase Nek7, alpha-1-antitrypsin, and zinc-alpha-2-glycoprotein; (*D*)serotransferrin) are shown as 3D images and as associated graph views of standardized abundances of the selected spots and spot locations (Fig. 5).

4. Discussion

Bladder cancer is one of the most common genitourinary cancers. More than 90% of bladder tumors are diagnosed as bladder TCC. Recent studies have indicated that diagnosing bladder TCC in its early stages might significantly increase the cure rate of the disease and prevent its further progression to invasive bladder cancer.[32] Thus, proteomic strategy offers a valuable tool to monitor potential bladder TCC markers for prognosis and diagnosis.

A

	ICy 3	ICy5 + Cy2
Gel1	Pool	TCC
Gel2	Pool	TCC
Gel3	Pool	TCC
Gel4	Pool	Normal
Gel5	Pool	Normal
Gel6	Pool	Normal

B

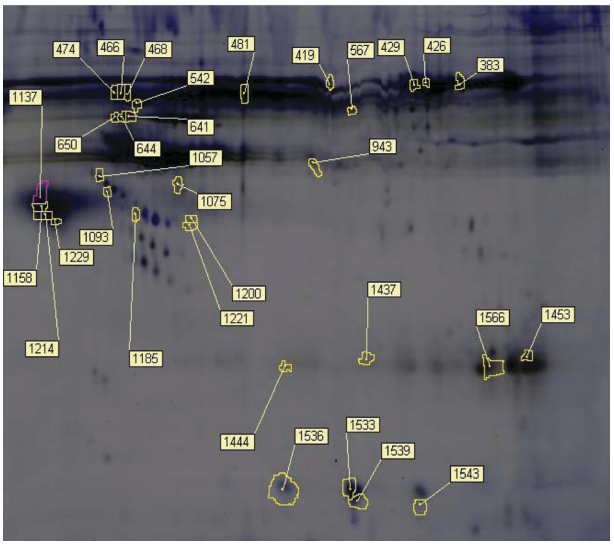

Fig. 4 Redox 2D-DIGE analysis of bladder TCC-induced differential cysteine-modification in plasma. (A) Plasma sample arrangement for a triplicated redox-2D-DIGE experiment. (B) Plasma samples (100 μg each) were labeled with ICy-dyes and separated using 24 cm, pH 3–10 non-linear IPG strips. 2D-DIGE images of the plasma samples from bladder TCC patients and healthy individuals at appropriate excitation and emission wavelengths were shown. Differentially labeled protein features are annotated with spot numbers.

Oxidative modification of proteins has been recognized as an important characteristic in cancer. Our previous study showed that cancer patients have significantly lower levels of total plasma thiol groups in comparison to healthy donors.[9] In contrast, cancer patients have been reported to have higher levels of plasma protein carbonylation than healthy donors.[12] Several lines of evidence also show that collagenase IV-associated tumor cell invasion is inhibited by reduced glutathione, implying that increased thiol oxidation consumes intracellular glutathione reservoirs and promotes tumor invasion.[33] Accordingly, decreased free thiol groups in cancer patients may have profound significance in cancer progression. Furthermore, the oxidized proteins in patient samples might be evaluated as disease markers for the disease.

Proteomic analyses of human diseases typically use a comparative method that is defined by the differential expression of the proteins under different disease states. Our 2D-DIGE/redox 2D-DIGE combined with MALDI-TOF MS analysis showed that 50 and 34 proteins displayed altered expression and thiol reactivity, respectively (Table 1 and 2). A majority of altered expressional proteins are located in the secreted fraction and belong to 5 major functional groups: inflammatory responses (55%), plasma protein transport (19%), blood coagulation (13%), cytoskeleton (9%), and associated regulation (9%). In contrast, the differentially thiol-reactive proteins were primarily secreted proteins and fell into 4 functional groups including cell adhesion (8%), inflammatory responses (50%), protease inhibitors (8%), and plasma protein transport (22%) (Fig. 6 and Table 2). Inflammatory response proteins are not only clearly modulated in protein levels, but significantly alter thiol reactivities in TCC patients' plasma (Table 1 and 2, and Fig. 6). Numerous plasma transport proteins were either regulated in the expression level (apolipoprotein A-I, carbonic anhydrase 1, hemoglobin, hemopexin, serum albumin, and serum amyloid P-component) or redox modified (apolipoprotein A-IV, hemopexin, serotransferrin, and serum albumin), correlating with bladder TCC patients having symptoms such as impaired cholesterol, heme iron, and lipoprotein transport.

The purpose of performing both lysine- and cysteine-based proteomic analyses is to monitor the differential levels in protein translation and in protein post-translation, respectively. The lysine-based proteomic analysis is to examine the total secreted protein changes contributed by bladder TCC. In contrast, the determination of thiol reactivity changes is to investigate the quality of plasma proteins with/without redox-alteration contributed by bladder TCC, as the redox-alteration is closely correlated with protein function. Notably, the redox-proteomic results are not affected by the protein level changes because a normalization of protein levels was performed before the calculation of thiol reactivity changes in our study. Thus, our current study can simultaneously monitor the changes of plasma protein levels and plasma protein functions in bladder TCC patients and healthy donors. Four identified proteins, including alpha-1-antitrypsin, haptoglobin, hemopexin, and leucine-rich alpha-2-glycoprotein were altered in both plasma protein levels and thiol reactivity, implying that the biological roles of these 4 proteins are significantly altered in bladder TCC. For example, downregulated or defected haptoglobin might reduce heme-recovery activity, followed by an increase in plasma oxidative stress through free heme in TCC plasma. This might also account for the high oxidative status of plasma in bladder TCC patients.

Our study is the first to use redox-proteomic strategy to monitor the bladder TCC-induced plasma protein oxidative damage. All identified redox-modulated proteins have not been reported as oxidative targets in bladder TCC-related research, suggesting that these oxidized proteins in patient samples might not only be evaluated as disease markers for bladder TCC, but elucidate the detail mechanisms in bladder TCC-associated redox-regulations. Further investigation shows that the combinations of these identified proteins have not yet been described as cancer markers in other cancer types. Accordingly, the combination of these identified proteins could be further evaluated as bladder TCC specific markers.

We have identified particular proteins, including alpha-1-antitrypsin,[34] alpha-1-antichymotrypsin,[35] haptoglobin,[36] and hemoglobin subunit beta,[37] which have been reported as bladder TCC marker proteins. Additionally, identified leucine-rich alpha-2-glycoprotein and fibrinogen beta chains have been monitored in nonmuscle invasive tumors of the bladder.[38] However, identified proteins, including apolipoprotein A-I, carbonic anhydrase 1, fibrinogen beta chain, hemopexin,

Table 2 Differentially labeled proteins identified by cysteine labeling 2D-DIGE and MS

Spot No.	Swissprot No.	Protein name	M_W	p*I*	No. match. peptides	Cov. (%)	Score	TCC/ normal (cys)[c]	TCC/ normal (lys)[c]	TCC/ normal (cys/lys)[c]	Functional classification	Match peptide
1137	P02763	Alpha-1-acid glycoprotein[a]	23 725	4.93	7/17	31	96/56	3.31	1.82	1.82	Inflammatory response	K.SDVVYTDWKK.D K.WFYIASAFR.N
1229	P02763	Alpha-1-acid glycoprotein[a]	23 725	4.93	7/18	31	104/56	2.49	1.19	2.09	Inflammatory response	K.DKCEPLEK.Q K.SDVVYTDWK.K
1146	P02763	Alpha-1-acid glycoprotein[a]	23 725	4.93	7/28	35	90/56	4.40	−1.58	5.54	Inflammatory response	K.TEDTIFLR.E K.SDVVYTDWK.K
1158	P02763	Alpha-1-acid glycoprotein[a,b]	23 725	4.93	7/30 (1/1)	31 (4)	76/56 (52/30)	3.30	−1.15	3.80	Inflammatory response	K.SDVVYTDWKK.D K.SDVVYTDWK.K (K.EGYYGYTGAFR.C)
943	Q86U18	Alpha-1-antitrypsin	46 878	5.37	9/49	27	63/56	1.20	1.98	−1.65	Inflammatory response	K.SVLGQLGITK.V K.SPLFMGKVVNPTQK.-
1075	P06727	Apolipoprotein A-IV	45 371	5.28	25/43	57	269/56	1.11	−5.42	−1.76	Transport	K.VNSFFSTFK.E K.EAVEHLQK.S
1200	P00738	Haptoglobin[a]	45 861	6.13	13/29	30	149/56	1.79	−1.17	2.60	Inflammatory response	K.DYAEVGR.V K.QLVEIEK.V
1214	P00738	Haptoglobin[a]	45 861	6.13	8/53	21	83/56	3.50	1.58	2.22	Inflammatory response	K.LPECEADDGCPKPPEIAHGYVEHSVR.Y K.LRTEGDGVYTLNNEK.Q
1221	P00738	Haptoglobin[a]	45 861	6.13	7/34	21	65/56	1.68	1.02	1.65	Inflammatory response	K.NYYKLR.T R.VGYVSGWGR.N
1533	P00738	Haptoglobin[a]	45 861	6.13	6/18	14	66/56	−2.02	1.48	−2.99	Inflammatory response	R.TEGDGVYTLNNEK.Q R.TEGDGVYTLNDK.K
1539	P00738	Haptoglobin[a]	45 861	6.13	10/37	19	93/56	−1.27	2.00	−2.54	Inflammatory response	R.TEGDGVYTLNDKK.Q K.LPECEAVCGKPK.N
1543	P00738	Haptoglobin[a]	45 861	6.13	6/18	10	64/56	−1.27	2.16	−2.74	Inflammatory response	K.NYYKLR.T R.TEGDGVYTLNDK.K
1536	P02790	Hemopexin	52 385	6.55	14/46	31	115/56	−1.32	2.17	−2.86	Transport	K.EVGTPHGIILDSVDAAFICPGSSR.L K.LLQDEFPGIPSPLDAAVECHR.G
542	P01876	Ig alpha-1 chain C region	38 486	6.08	8/37	27	78/56	1.56	−1.02	1.59	Inflammatory response	K.SAVQGPPER.D K.YLTWASR.Q
1437	P01835	Ig kappa chain C region[a]	11 773	5.58	7/35	88	114/56	−2.44	−1.75	−1.62	Inflammatory response	K.DSTYSLSSTLTLSK.A K.SGTASVVCLLNNFYPR.E
1453	P01835	Ig kappa chain C region[a]	11 773	5.58	5/36	67	67/56	−2.63	−1.17	1.52	Inflammatory response	K.SFNRGEC.-K.SGTASVVCLLNNFYPR.E
1566	P01835	Ig kappa chain C region[a]	11 773	5.58	4/26	54	66/56	−2.02	−1.31	−1.32	Inflammatory response	K.SGTASVVCLLNNFYPR.E K.VYACEVTHQGLSSPVTK.S
641	P01042	Kininogen-1[a]	72 996	6.34	9/21	12	76/56	2.50	1.53	1.63	Protease inhibitor	K.ICVGCPR.D R.QVVAGLNFR.I
644	P01042	Kininogen-1[a]	72 996	6.34	15/35	20	147/56	3.73	2.22	1.68	Protease inhibitor	R.IASFSQNCDIYPGKDFVQPPTK.I K.KYNSQNQSNNQFVLYR.I
650	P01042	Kininogen-1[a]	72 996	6.34	12/34	18	98/56	2.38	1.57	1.52	Protease inhibitor	R.IASFSQNCDIYPGKDFVQPPTK.I R.DIPTNSPELEETLTHTITK.L
1057	P02750	Leucine-rich alpha-2-glycoprotein	38 386	6.45	5/11	13	64/56	2.88	1.32	2.18	Inflammatory response	R.GPLQLER.L K.DLLLPQPDLR.Y
534	Q6MZT7	Protein THEMIS	74 044	5.62	6/9	8	68/56	1.67	−1.24	2.08	T-cell development	R.HHVDITKK.L K.QFRLPFNVK.V
1093	Q8TDX7	Serine/threonine-protein kinase Nek7	34 985	8.49	6/22	26	71/56	2.98	1.79	1.66	Cell cycle	K.QLNHPNVIK.Y K.VQIFDLMDAK.A
426	P02787	Serotransferrin[a]	79 294	6.81	22/49	26	218/56	1.31	−1.43	1.87	Transport	R.FDEFFSEGCAPGSK.K K.CDEWSVNSVGK.I
429	P02787	Serotransferrin[a]	79 294	6.81	7/18	10	71/56	1.50	−1.37	2.06	Transport	K.EGYYGYTGAFR.C R.FDEFFSEGCAPGSK.K
474	P02787	Serotransferrin[a]	79 294	6.81	24/52	35	188/56	1.79	1.13	1.58	Transport	K.HSTIFENLANK.A K.EDPQTFYYAVAVVK.K
383	P02787	Serotransferrin[a,b]	79 294	6.81	6/21 (1/1)	8(1)	70/56 (46/30)	−1.39	1.57	−2.18	Transport	K.DSGFQMNQLR.G K.EGYYGYTGAFR.C11 (K.EGYYGYTGAFR.C)
567	Q9P157	Serum albumin[a]	71 317	5.92	13/25	20	117/56	1.90	−1.12	−2.13	Transport	K.AEFAEVSK.L R.DAHKSEVAHR.F
1536	Q9P157	Serum albumin[a]	71 317	5.92	19/46	31	152/56	−1.32	2.17	−2.86	Transport	K.VHTECCHGDLLECADDRADLAK.Y K.KLVAASQAALGL.-

Table 2 *(continued)*

Spot No.	Swissprot No.	Protein name	M_w	pI	No. match. peptides	Cov. (%)	Score	TCC/ normal (cys)[c]	TCC/ normal (lys)[c]	TCC/ normal (cys/lys)[c]	Functional classification	Match peptide
419	Q9NV66	*t*RNA wybutosine-synthesizing protein 1 homolog	84751	6.42	5/7	5	57/56	1.42	−1.28	1.82	Biosynthesis	K.TQGKNLQEK.S K.DIFVSGVK.I
466	P04004	Vitronectin[a]	55069	5.55	12/39	19	78/56	1.85	1.14	1.62	Cell adhesion	R.SQRGHSR.G R.SQRGHSR.G
468	P04004	Vitronectin[a]	55069	5.55	13/36	24	108/56	1.76	1.11	1.59	Cell adhesion	R.DVWGIEGPIDAAFTR.I R.QPQFISR.D
474	P04004	Vitronectin[a]	55069	5.55	11/21	22	111/56	1.79	1.13	1.58	Cell adhesion	R.QPQFISR.D K.AVRPGYPK.L
1184	P25311	Zinc-alpha-2-glycoprotein	34469	5.71	11/46	30	95/56	2.81	−1.39	3.91	Lipid degradation	R.EDIFMETLK.D K.IDVHWTR.A

Plasma proteins displaying differential ICy dye labeling between bladder TCC patients and healthy subjects were identified by both MALDI-TOF peptide mass mapping analysis and MALDI-TOF/TOF sequence analysis. [a] Proteins appearing more than once were identified as isoforms with different percentage coverage of analyzed peptides, matched peptide numbers, MOWSE scores, average fold-thiol reactivity alterations, *t*-test values, and matched peptide sequences for each identified protein. [b] These proteins have been identified by both MALDI-TOF MS and MALDI-TOF/TOF MS. The parameters (sequence coverages, MASCOT values, matched peptide numbers, and matched peptide sequences) shown in brackets are obtained from MALDI-TOF/TOF MS sequence analysis. [c] Average fold-differences of triplicate samples run on different gels from DeCyder analysis show labeling changes on the free thiol group of cysteine residues for bladder TCC plasma *versus* healthy donor's plasma. Here, ICy5 signals were used to monitor cysteine-labeling alterations against ICy3 signals, and used as an internal standard to follow normalized with protein expression changes. Additionally, NHS-Cy2 signals were used to monitor lysine-labeling alterations (protein expression changes) against ICy3 signals, and were used as an internal standard. Proteins displaying an average fold-difference of 1.5-fold up- or down-regulation are shown in the table. Functions were adopted from the Swiss-Prot database and research.

Fig. 5 Representative images of the identified spots ((A) serine/threonine-protein kinase Nek7; (B) alpha-1-antitrypsin; (C) zinc-alpha-2-glycoprotein; (D) serotransferrin) displaying bladder TCC-dependent protein thiol reactivity changes. The thiol reactivities of these proteins are visualized as 2-DE fluorescent images (top panels), 3D spot images (middle panels), and protein thiol reactive maps (bottom panels).

Fig. 6 Percentage of identified differentially expressed plasma proteins (A) and thiol reactive plasma proteins (B) between bladder TCC cases and healthy donors according to their sub-cellular locations (left panels) and biological functions (right panels) by 2D-DIGE/MALDI-TOF MS.

leucine-rich alpha-2-glycoprotein, selenocysteine-specific elongation factor, and serum amyloid P-component, have not been reported as bladder TCC markers, and require further validation as potential bladder TCC biomarkers. Apolipoprotein A-I is a component of the high density lipoprotein responsible for cholesterol transport in plasma.[39] In numerous cancers, such as oesophageal cancer, cholangiocarcinoma, colon cancer, and ovarian cancer, the apolipoprotein A-I level is up-regulated;[40–44] however, reduced plasma apolipoprotein A-I level is associated with gastric tumors.[45] Our current quantitative analysis showed the apolipoprotein A-I level is down-regulated in bladder TCC. These studies and our results show that the regulation of the apolipoprotein A-I level during tumorigenesis is complicated and requires further clarification. In addition, leucine-rich alpha-2-glycoprotein has been shown to be involved in cell adhesion, and has been recognized as a biomarker for certain diseases, including microbial infections, ovarian cancer, and pancreatic cancer.[46,47] Our proteomic result is consistent with these investigations and is the first to support leucine-rich alpha-2-glycoprotein as a potential signature for bladder TCC.

Isolation of low-abundance proteins from patient plasma is frequently complicated because of the existence of high abundance proteins such as immunoglobulin and serum albumin. Serum albumin is the most (50%–70%), and immunoglobulin is the second most (10%–20%), abundant protein in plasma. These proteins mask low-abundant proteins, and restrict the amount of the total plasma proteins that can be applied and resolved by proteomic analysis. To remove these 2 high-abundant proteins, we used an albumin and IgG depletion kit from Sigma-Aldrich, which contains prepacked spin columns, allowing removal of the majority of both immunoglobulin and serum albumin. We used trichloroacetic acid/acetone to precipitate, desalt, and enrich the plasma proteins to render them well resolved by 2D-DIGE. Our results showed that this strategy enabled the plasma proteins to be well prepared and separated (data not shown). Our proteomic analysis showed that serum albumin showed a 1.69-fold reduced level in TCC plasma in comparison with plasma from healthy donors. This value did not consider the process of albumin-depletion, implying that a measurement deviation might occur between the 1.69-fold reduced level and the exacted albumin level in whole blood; nevertheless, the depletion efficiency should be identical for TCC plasma and healthy plasma, suggesting a significant down-regulation of serum albumin in bladder TCC.

Our 2D-DIGE experiment used fluorescence-based quantitation, which is able to detect sub-nanograms in dye-labeled proteins; however, our post-staining experiment used colloidal Coomassie blue staining with sensitivity in the 10–50 nanogram range.[48] Thus, numerous differentially expressed dye-labeled low-abundant plasma proteins can be visualized by a fluorescent scanner but were undetected with colloidal Coomassie blue staining. This is why only 50 out of 101 (experiments performed with minimal Cy-dye labeling) and 34 out of 174 (experiments performed with saturated ICy-dye labeling) differentially expressed and thiol reactivity changed features on 2-DE, respectively, can be chosen and successfully identified by MALDI-TOF/MALDI-TOF/TOF analysis. In addition, the plasma proteins are largely glycosylated in the circulation system. The high level of glycosylation on plasma proteins have shown to interfere with trypsin digestion and MALDI-TOF MS analysis.[49] Consequently, glycosylation of plasma proteins might contribute to a low-identification rate in this study.

Our quantitative cysteine- and lysine-2D-DIGE combining MALDI-TOF MS analysis has identified previously reported plasma markers of bladder TCC, such as haptoglobin, and identified a panel of unreported plasma proteins found to be differentially-altered between bladder TCC patients and healthy donors in protein abundance and thiol reactivity, such as leucine-rich alpha-2-glycoprotein. These newly-defined bladder TCC markers have potential in evaluating their usages in both bladder TCC diagnosis and eventual prognosis.

5. Declaration of competing interests

The authors confirm that there are no conflicts of interest.

Abbreviations

2-DE	two-dimensional gel electrophoresis
Ab	antibody
CCB	colloidal coomassie blue
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate)
ddH_2O	double deionized water
DIGE	differential gel electrophoresis
DTT	dithiothreitol
MALDI-TOF MS	matrix assisted laser desorption ionization–time of flight mass spectrometry
TCC	transitional cell carcinoma
TFA	trifluoroacetic acid

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References

1 T. F. Wu, W. L. Ku and Y. G. Tsay, *Expert Rev. Proteomics*, 2007, **4**, 639–647.
2 S. Sengupta and M. L. Blute, *Urology*, 2006, **67**, 48–54.
3 R. S. Lance and H. B. Grossman, *Adv. Exp. Med. Biol.*, 2003, **539**, 3–14.
4 H. B. Grossman and C. P. Dinney, *Urol. Oncol.*, 2000, **5**, 3–10.
5 Y. C. Tyan, M. H. Yang, S. C. Chen, S. B. Jong, W. C. Chen, Y. H. Yang, T. W. Chung and P. C. Liao, *Rapid Commun. Mass Spectrom.*, 2011, **25**, 2851–2862.
6 M. S. Soloway, V. Briggman, G. A. Carpinito, G. W. Chodak, P. A. Church, D. L. Lamm, P. Lange, E. Messing, R. M. Pasciak, G. B. Reservitz, D. B. Rukstalis, M. F. Sarosdy, W. M. Stadler, R. P. Thiel and C. L. Hayden, *J. Urol.*, 1996, **156**, 363–367.
7 B. S. Schmetter, K. K. Habicht, D. L. Lamm, A. Morales, N. H. Bander, H. B. Grossman, M. G. Hanna, Jr., S. R. Silberman and B. T. Butman, *J. Urol.*, 1997, **158**, 801–805.
8 Y. C. Tyan, M. H. Yang, S. C. Chen, S. B. Jong, W. C. Chen, Y. H. Yang, T. W. Chung and P. C. Liao, *Rapid Commun. Mass Spectrom.*, 2011, **25**, 2851–2862.
9 I. A. Yilmaz, T. Akcay, U. Cakatay, A. Telci, S. Ataus and V. Yalcin, *Int. Urol. Nephrol.*, 2003, **35**, 345–350.

10 R. T. Dean, S. Fu, R. Stocker and M. J. Davies, *Biochem. J.*, 1997, **324**(Pt 1), 1–18.
11 B. S. Berlett and E. R. Stadtman, *J. Biol. Chem.*, 1997, **272**, 20313–20316.
12 S. Popadiuk, M. Korzon, J. Renke and M. Wozniak, *Wiad. Lek.*, 1998, **51**(suppl. 4), 107–112.
13 B. Pignatelli, C. Q. Li, P. Boffetta, Q. Chen, W. Ahrens, F. Nyberg, A. Mukeria, I. Bruske-Hohlfeld, C. Fortes, V. Constantinescu, H. Ischiropoulos and H. Ohshima, *Cancer Res.*, 2001, **61**, 778–784.
14 R. F. della, A. Granata, A. Saija, M. Broccio, A. Tomaino, A. Zirilli, G. De Caridi and G. Broccio, *Anticancer Res.*, 2000, **20**, 1595–1598.
15 E. Beutler and T. Gelbart, *J. Lab. Clin. Med.*, 1985, **105**, 581–584.
16 B. R. Herbert, J. L. Harry, N. H. Packer, A. A. Gooley, S. K. Pedersen and K. L. Williams, *Trends Biotechnol.*, 2001, **19**, 3–9.
17 T. Rabilloud, *Electrophoresis*, 1994, **15**, 278–82.
18 H. L. Huang, H. W. Hsing, T. C. Lai, Y. W. Chen, T. R. Lee, H. T. Chan, P. C. Lyu, C. L. Wu, Y. C. Lu, S. T. Lin, C. W. Lin, C. H. Lai, H. T. Chang, H. C. Chou and H. L. Chan, *J. Biomed. Sci.*, 2010, **17**, 36.
19 T. C. Lai, H. C. Chou, Y. W. Chen, T. R. Lee, H. T. Chan, H. H. Shen, W. T. Lee, S. T. Lin, Y. C. Lu, C. L. Wu and H. L. Chan, *J. Proteome Res.*, 2010, **9**, 1302–1322.
20 H. L. Chan, S. Gharbi, P. R. Gaffney, R. Cramer, M. D. Waterfield and J. F. Timms, *Proteomics*, 2005, **5**, 2908–2926.
21 Y. W. Chen, H. C. Chou, P. C. Lyu, H. S. Yin, F. L. Huang, W. S. Chang, C. Y. Fan, I. F. Tu, T. C. Lai, S. T. Lin, Y. C. Lu, C. L. Wu, S. H. Huang and H. L. Chan, *Funct. Integr. Genomics*, 2011, **11**, 225–239.
22 H. C. Chou, Y. W. Chen, T. R. Lee, F. S. Wu, H. T. Chan, P. C. Lyu, J. F. Timms and H. L. Chan, *Free Radical Biol. Med.*, 2010, **49**, 96–108.
23 H. L. Chan, P. R. Gaffney, M. D. Waterfield, H. Anderle, M. H. Peter, H. P. Schwarz, P. L. Turecek and J. F. Timms, *FEBS Lett.*, 2006, **580**, 3229–3236.
24 H. L. Chan, S. Gharbi, P. R. Gaffney, R. Cramer, M. D. Waterfield and J. F. Timms, *Proteomics*, 2005, **5**, 2908–2926.
25 H. L. Chan, S. Gharbi, P. R. Gaffney, R. Cramer, M. D. Waterfield and J. F. Timms, *Proteomics*, 2005, **5**, 2908–2926.
26 P. H. Hung, Y. W. Chen, K. C. Cheng, H. C. Chou, P. C. Lyu, Y. C. Lu, Y. R. Lee, C. T. Wu and H. L. Chan, *Mol. BioSyst.*, 2011, **7**, 1990–1998.
27 C. P. Lin, Y. W. Chen, W. H. Liu, H. C. Chou, Y. P. Chang, S. T. Lin, J. M. Li, S. F. Jian, Y. R. Lee and H. L. Chan, *Mol. BioSyst.*, 2011, **8**, 1136–1145.
28 Y. W. Chen, J. Y. Liu, S. T. Lin, J. M. Li, S. H. Huang, J. Y. Chen, J. Y. Wu, C. C. Kuo, C. L. Wu, Y. C. Lu, Y. H. Chen, C. Y. Fan, P. C. Huang, C. H. Law, P. C. Lyu, H. C. Chou and H. L. Chan, *Mol. BioSyst.*, 2011, **7**, 3065–3074.
29 S. P. Wolff, Z. Y. Jiang and J. V. Hunt, *Free Radical Biol. Med.*, 1991, **10**, 339–352.
30 I. A. Yilmaz, T. Akcay, U. Cakatay, A. Telci, S. Ataus and V. Yalcin, *Int. Urol. Nephrol.*, 2003, **35**, 345–350.
31 H. L. Chan, S. Gharbi, P. R. Gaffney, R. Cramer, M. D. Waterfield and J. F. Timms, *Proteomics*, 2005, **5**, 2908–2926.
32 J. T. Leppert, O. Shvarts, K. Kawaoka, R. Lieberman, A. S. Belldegrun and A. J. Pantuck, *Eur. Urol.*, 2006, **49**, 226–234.
33 S. Sheela and J. C. Barrett, *Carcinogenesis*, 1985, **6**, 173–179.
34 J. Sinard, L. Macleay, Jr. and J. Melamed, *Cancer*, 1994, **73**, 1919–1925.
35 J. Albores-Saavedra, F. Vuitch, R. Delgado, E. Wiley and H. Hagler, *Am. J. Surg. Pathol.*, 1994, **18**, 83–90.
36 H. Li, C. Li, H. Wu, T. Zhang, J. Wang, S. Wang and J. Chang, *Proteome Sci.*, 2011, **9**, 21.
37 J. Bellmunt, T. K. Choueiri, R. Fougeray, F. A. Schutz, Y. Salhi, E. Winquist, S. Culine, M. H. von der, D. J. Vaughn and J. E. Rosenberg, *J. Clin. Oncol.*, 2010, **28**, 1850–1855.
38 M. Linden, S. B. Lind, C. Mayrhofer, U. Segersten, K. Wester, Y. Lyutvinskiy, R. Zubarev, P. U. Malmstrom and U. Pettersson, *Proteomics*, 2012, **12**, 135–144.
39 D. J. Rader, *Am. J. Cardiol.*, 2003, **92**, 42J–49J.
40 Y. T. Chen, C. L. Chen, H. W. Chen, T. Chung, C. C. Wu, C. D. Chen, C. W. Hsu, M. C. Chen, K. H. Tsui, P. L. Chang, Y. S. Chang and J. S. Yu, *J. Proteome Res.*, 2010, **9**, 5803–5815.
41 P. Kelly, V. Appleyard, K. Murray, F. Paulin, D. Lamont, L. Baker, S. Suttie, D. Exon and A. Thompson, *Br. J. Cancer*, 2010, **103**, 232–238.
42 X. Wang, S. Dai, Z. Zhang, L. Liu, J. Wang, X. Xiao, D. He and B. Liu, *Eur. J. Cancer*, 2009, **18**, 625–635.
43 K. R. Kozak, F. Su, J. P. Whitelegge, K. Faull, S. Reddy and R. Farias-Eisner, *Proteomics*, 2005, **5**, 4589–4596.
44 M. Tachibana, Y. Ohkura, Y. Kobayashi, H. Sakamoto, Y. Tanaka, J. Watanabe, K. Amikura, Y. Nishimura and K. Akagi, *Anticancer Res.*, 2003, **23**, 4161–4167.
45 P. K. Chong, H. Lee, J. Zhou, S. C. Liu, M. C. Loh, J. B. So, K. H. Lim, K. G. Yeoh and Y. P. Lim, *J. Proteomics*, 2010, **73**, 1632–1640.
46 J. D. Andersen, K. L. Boylan, R. Jemmerson, M. A. Geller, B. Misemer, K. M. Harrington, S. Weivoda, B. A. Witthuhn, P. Argenta, R. I. Vogel and A. P. Skubitz, *J. Ovarian Res.*, 2010, **3**, 21.
47 T. Kakisaka, T. Kondo, T. Okano, K. Fujii, K. Honda, M. Endo, A. Tsuchida, T. Aoki, T. Itoi, F. Moriyasu, T. Yamada, H. Kato, T. Nishimura, S. Todo and S. Hirohashi, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2007, **852**, 257–267.
48 H. L. Chan, S. Gharbi, P. R. Gaffney, R. Cramer, M. D. Waterfield and J. F. Timms, *Proteomics*, 2005, **5**, 2908–2926.
49 D. S. Dalpathado and H. Desaire, *Analyst*, 2008, **133**, 731–738.