Original Paper

Gene Microarray Integrated with High-Throughput Proteomics for the Discovery of Transthyretin in Rhabdomyolysis-Induced Acute Kidney Injury

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Key Words

Transthyretin • Rhabdomyolysis • Apoptosis

Abstract

Background/Aims: Rhabdomyolysis, one of the leading causes of acute kidney injury (AKI), develops after trauma, drug toxicity, infections, burns, and physical exertion. The aim of this study was to investigate differences in gene and protein expression to elucidate the pathogenesis of rhabdomyolysis (RM)-induced AKI. **Methods:** In this study, we used glycerol induced renal injury as a model of RM-induced AKI. Affymetrix U133 plus 2.0 microarrays were used to perform gene microarray analysis. Isobaric tagging with related and absolute quantitation (iTRAQ) labeling mass spectrometry (MS) was applied to screen and identify differentially expressed proteins between RM-induced AKI and normal murine renal tissue. Verification experiments included immunohistochemistry (IHC), real-time PCR, Western blotting, and the measurement of ATP and ROS production. HK-2 cells were incubated *in vitro* with ferrous myoglobin and pcDNA-TTR, followed by assays to detect cell proliferation, ROS and apoptosis. **Results:** According to gene microarray and iTRAQ-MS analysis, we screened 17 common elements. After multiple analyses, we selected transthyretin (TTR) as our focus and investigated TTR in the kidney. Verification experiments with IHC confirmed differential expression levels of TTR proteins. Furthermore, Western blotting showed a stepwise decrease in TTR in AKI renal tissues. Cell-based experiments showed that overexpression of TTR could improve HK-2 cell viability and inhibit apoptosis. TTR reduced apoptosis by decreasing the accumulation of reactive oxygen species (ROS). **Conclusion:** This study reports a possible mechanism for RM-induced AKI and suggests that reductions in TTR could increase the generation of ROS and induce apoptosis. TTR may be a potentially valuable target for RM-induced AKI.

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Published by S. Karger AG, Basel

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Introduction

Acute kidney injury (AKI) is a clinical syndrome that occurs within 48 h of abnormal renal function or structure. AKI is a severe complication of rhabdomyolysis (RM), which is common following compression, exercise, drug use and infection. In these cases, skeletal muscle destruction and the release of their intracellular content into the circulation, such as myoglobin, creatine kinase, and lactate dehydrogenase, result in fluid-electrolyte imbalance, acid-base disturbances, coagulopathies and renal dysfunction [1]. Approximately 15-40% of rhabdomyolysis cases can lead to AKI. Without early diagnosis and treatment, AKI can develop into multiple organs dysfunction syndrome (MODS) with high mortality [2].

Although the pathophysiologic mechanisms of RM-induced AKI are not yet completely understood, some studies have shown that a large amount of myoglobin from destroyed striated muscle is released into the blood, which exceeds the threshold of renal excretion and blocks renal tubules. Myoglobin in acidic conditions decomposes into globin and heme, with the latter forming reactive oxygen species (ROS) that can damage renal tubules [3]. In the process of AKI, renal tubular cell injury is always associated with tubular apoptosis, which is different from traditional necrosis [4]. Therefore, early detection and individual treatment are of great significance for RM. The screening and identification of new genes or proteins is needed to better diagnose, treat, predict and monitor the recurrence of RM.

As the products of gene transcription and translation, proteins are responsible for cellular phenotypes. So far, there have been few integrative analyses of proteomic and genomic data for AKI, including RM, highlighting the urgent need to integrate proteomics with genomics to obtain a more comprehensive understanding of bioinformatics flow in RM-induced AKI.

To study the pathogenesis of RM-induced AKI, we applied gene microarray integrated with high-throughput proteomics to screen and investigate changes in gene and protein expression in RM-induced AKI. Affymetrix U133 plus 2.0 microarrays were used for the gene microarray analysis. Isobaric tagging with related and absolute quantitation (iTRAQ) labeling mass spectrometry (MS) was used as the proteomic tool. We found that transthyretin, both at the protein and gene level, was significantly altered in RM-induced AKI.

Transthyretin (TTR), also known as prealbumin, is composed of four identical tetrameric subunits found mainly in the liver, choroid plexus and during retinal synthesis [5]. TTR is classically involved in the transport of thyroxine and retinol. TTR can also accurately reflect the body's internal protein synthesis, and as such can be used to assess the nutritional status of patients [6]. In addition, TTR is closely associated with familial amyloidosis, ophthalmic diseases, cardiovascular diseases, nervous system diseases and the occurrence of various systemic tumors, but relevant research has not been reported with regard to acute renal injury. As such, this study aimed to explore the potential mechanism by which TTR mediates RM-induced AKI.

Materials and Methods

Experimental design and analytical strategy

Briefly, there were three consecutive phases in this study. First, a discovery screen of differential gene expression microarray analysis and quantitative proteomics based on iTRAQ was performed to identify common elements. Second, a protein level evaluation of promising genes and proteins was assessed by Western blotting and immunohistochemistry. Third, a validation of the candidate genes and proteins by cytological studies was performed. This study was designed and performed in accordance with the Guide for the Care and Use of Laboratory Animals of the Chinese PLA General Hospital and Military Medical College. The research protocols were approved by the Animal Ethics Committee of the Chinese PLA General Hospital and Military Medical College. All methods were performed in accordance with approved guideline.

Experimental animals and procedures

We established an RM-induced mouse AKI model for proteomics analysis. Eight- to twelve-week-old C57BL/6 male mice were provided by the Experimental Animal Center of the Academy of Military Medical Sciences. The mice were housed at a stable temperature and a 12 h light/dark cycle. We provided adequate standard rodent chow and water. The animals were acclimated for seven days before initiating the experiment. All animal protocols were approved by the Animal Ethics Committee of the Chinese PLA General Hospital and Military Medical College. C57BL/6 mice were deprived of water for 24 h and then administered half the dose of glycerol (50% v/v in sterile saline) in each hindlimb muscle following mild sedation with pentobarbital. Our dose-dependent studies defined the optimal dose of glycerol as 8 mL/kg body weight. Sixteen male mice were randomly assigned to either the control group (Con, n = 8) or the RM-induced AKI group (RM, n = 8). The blood, urine and kidney tissue were harvested for further processing 72 h after induction of rhabdomyolysis.

Histopathological examination of acute tubular necrosis scores

The kidneys were fixed in 10% v/v formalin for 24 h followed by routine dehydration and paraffin embedding. Renal tissues were sectioned at 3- μ m thickness and stained with periodic acid-Schiff (PAS) using standard methods [7]. Histological examinations were performed in a blinded fashion. Acute tubular necrosis severity was quantified by counting the percentage of tubules that displayed cell necrosis, loss of brush border, cast formation and tubule dilation as follows: 0 = none, 1 = $\leq$ 10%; 2 = 11% to 25%; 3 = 26% to 45%; 4 = 46% to 75%; and 5 = $>$ 76%[8]. Approximately 20 high-power fields (HPFs, $\times$ 200) per individual mouse (10 HPFs per slide, 6 slides per animal) were evaluated (n = 6 per group)

Urine collection and measurement of urinary Kim-1

All of the collected urine samples were centrifuged for 5 min at 10,000 r/min. Supernatants were collected, aliquoted, and stored at -80°C until further analysis. Kim-1 was measured in the urine collected from mice by enzyme-linked immunoassay. The urinary supernatants were used for the measurement of Kim-1 using an ELISA kit (R&D Systems, Minneapolis, MN)

Data sources for gene analysis

Our gene data and related information can be accessed at the Gene Expression Omnibus database. The accession number is GSE44925. The data collection method for gene analysis was based on our previous report [9]. Briefly, 3 untreated mice and 5 mice with rhabdomyolysis were selected for gene microarray analysis. We intramuscularly injected 50% v/v glycerol (0.05 mL per 10 g body weight) into the left hind limb under inhalation anesthesia to induce AKI.

Differential gene expression data in RM-induced AKI

The genes differentially expressed between normal and RM-induced AKI tissues were identified using the TwoClassDif method and Gene Ontology (GO)-Analysis. Affymetrix U133 plus 2.0 microarrays were used to perform the gene microarray analysis. According to the Random Variance Model (RVM) t-test, we filtered the differentially expressed genes between the normal tissue and AKI groups. The RVM t-test was applied to filter the differentially expressed genes for the control and experimental groups because the RVM t-test can increase the degrees of freedom in small sample data sets. We selected the differentially expressed genes according to the P-value threshold after significance analysis and FDR analysis were performed. A P-value $<$ 0.05 was considered significant [10-12]. Furthermore, the differentially expressed genes were subjected to multiple testing corrections (Benajmini-Hochberg correction) to decrease the number of false positives.

Protein preparation and iTRAQ labeling

The protein samples were quantitated by the Bradford method. The iTRAQ labeling was performed according to the protocol. Briefly, 200 μ g protein was precipitated at -20°C for 60 min. Then, precipitated protein was resuspended in 20 μ L dissolution buffer. After reduction and alkylation, the peptides were labeled with iTRAQ reagents for 60 min. Six iTRAQ reagents were used to label the normal and AKI peptides. The samples were mixed together sequentially and desalted with Sep-Pak Vac C18 cartridges.

LC-MS/MS analysis

The mixed labeled peptides were fractionated by strong cation exchange chromatography (SCX). The mixture was reconstituted with Buffer A (10 mM KH_2PO_4 in 25% v/v acetonitrile, pH 2.6). The mixed peptides were separated at a 500 $\mu\text{L}/\text{min}$ with a gradient of 0–80% Buffer B (Buffer B was Buffer A containing 350 mM KCl) in Buffer A for 1 h. The 215 nm and 280 nm absorbance values were monitored, and a total of 12 SCX fractions were pooled. The fractions were dried and resuspended in 50 μL HPLC Buffer A (5% v/v acetonitrile, 0.1% v/v formic acid). Then, samples were loaded across the Zorbax 300 SBC18 reverse-phase column and assessed on a QSTARXL System with a 20 AD HPLC system. The elution flowrate was 0.3 $\mu\text{L}/\text{min}$ with a gradient of 5 %–35 % v/v HPLC Buffer B (98% v/v acetonitrile, 0.1% v/v formic acid) for 90 min. The scans were obtained with m/z ranges of 400–1800 for MS with up to three precursors selected from m/z 100–2000 for MS/MS.

Mass spectrometry data analysis

Protein identification was performed using Protein Pilot 4.2 beta (ABSciex, Framingham, MA, USA). The Paragon algorithm in Protein Pilot software served as the default search program with trypsin as the digestion agent and MMTS as a fixed modification of cysteine. Biological modifications were programmed in the algorithm. Each MS/MS spectrum was searched in the SWISSPROT database for *Homo sapiens*. Proteins were identified with a global FDR of less than 5%.

Immunohistochemistry

Immunohistochemical staining for the detection of TTR in renal tissue was performed on formaldehyde-fixed and paraffin-embedded tissues using the avidin-biotin-immunoperoxidase method. Kidney sections were subjected to antigen retrieval, and sections were blocked and labeled overnight at 4°C with rabbit anti-Prealbumin (Transthyretin, TTR) (1:100 v/v, Abcam, Cambridge, MA, USA). After incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (Dako, Glostrup, Denmark), the sections were treated with avidin-biotin-peroxidase conjugate (ABC Kit, Vector Laboratories, Burlingame, CA, USA). The reaction was visualized using a 3, 3'-diaminobenzidine (DAB) chromogen (Dako) following tissue counterstaining with hematoxylin.

Real-time PCR

The total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA). A TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and a Gene Amp® PCR System 9700 platform (Applied Biosystems) were used to generate cDNA. The gene expression analysis was conducted by quantitative real-time PCR using SYBR Green Mastermix and a 7500 Real-time PCR System (Applied Biosystems). The results were analyzed using the $2^{-\Delta\Delta\text{CT}}$ method and were normalized ($n = 6$) to RM-induced renal injury ($n = 6$). The primers are shown in Table 1.

Western blot analysis

Rabbit polyclonal anti-Transthyretin and anti-cleaved caspase-3 horseradish peroxidase labeled goat anti-rabbit immunoglobulin G (IgG), as well as rabbit anti-mouse IgG, were purchased from Abcam (Abcam, Cambridge, MA, USA). Mouse anti- β -actin monoclonal antibody was obtained from Sigma (St. Louis, MO, USA). Frozen

kidney tissue was homogenized for radio-immunoprecipitation. The homogenates were clarified by centrifugation at 12,000 g for 10 min at 4°C, and the supernatants were collected. Supernatant samples (70 μg) were subjected to 15% v/v polyacrylamide

Table 1. Gene names and the primers in Real-time PCR

Gene Name	Fw(5'→3')	Rev(5'→3')	Products length
Afm	CACGACATACCTGGCCATCA	GTTGTGAGCCAGACCTAT	118 bp
Cfi	TGTAATGGTGAGGTGGACTGC	GTGCAAGGCCTTTTGGAATGT	88 bp
Ttr	ACCGGTGAATCCAAGTGTCC	CTCCAGGTGTCATCAGCAG	114bp
Psm5	ACCAACATCGAGCTAGCCAC	TCGAGGACGGCTCCTTCTTA	107 bp
Vcl	TGGTCTAGCAAGGGCAATGA	ACACTGAATAAGTGCCCGCT	117 bp
Klf10	TCTGACATGCAAACTTGAACCC	TTGTGCAAATTGTAACCTCGTGA	78 bp
Klf12	TACAGTCAGTGCCGGTTGTC	GCTCCATTGTGCTTTGCCA	114 bp
GAPDH	AATTGAGCCCGCAGCTCCC	CCAGGCGCCAATACGACCA	139bp

gel electrophoresis and transferred to cellulose acetate membranes. The membranes were blocked with 1×casein solution for approximately 4 h and then incubated with rabbit polyclonal anti-Transthyretin and anti-cleaved caspase-3 overnight at 4°C. The blots were washed with Tris-buffered saline-Tween-20 (TBST) buffer and subsequently incubated with goat anti-rabbit or rabbit anti-mouse IgG. After washing with TBST, the blots were developed with enhanced chemiluminescence reagents. A mouse monoclonal anti-β-actin antibody was used as a control for each sample. Blot signals were analyzed by the free image analyzing software ImageJ 1.42 (downloaded from <http://rsbweb.nih.gov/ij/download.html>).

Determination of ROS production in kidney tissue and ATP measurement

Mouse kidney homogenates were analyzed fluorometrically by measuring the oxidation of the non-fluorescent probe 20, 70-dichloro-fluorescein diacetate (DCFDA) in the fluorescent metabolite DCF as described by Topo et al. [13]. In brief, 30 mL of kidney homogenate in PBS was mixed with 5 μM DCFDA and incubated for 30 minutes at room temperature. The mean fluorescence intensity was directly measured at excitation and emission wavelengths of 485 and 535 nm, respectively.

Renal cortical tissue ATP was measured as described by Vives-Bauza et al. [14]. ATP was extracted from flash frozen kidney cortex with 0.4 M HClO₄. ATP levels were determined using an ATP bioluminescence assay kit (Roche) and normalized to the protein concentration.

TTR overexpression plasmid construction and transfection

A full-length TTR clone was purchased from Youbio (Changsha, China) and cloned into pcDNA3.1(+) to make pcDNA-TTR. After confirming the DNA sequence, pcDNA-TTR was transfected into HK-2 cells with jetPRIME reagent (Polyplus transfection) with pcDNA3.1(+) as the vehicle.

HK-2 cell culture and ferrous myoglobin media

Myoglobin (M0630, Sigma Aldrich Corporation, St. Louis, MO, USA) and ascorbic acid (Sigma) were dissolved in Dulbecco's Modified Eagle Medium (DMEM):Nutrient Mixture F-12 media (DMEM/F12) (Gibco, Life Technologies Corporation, Carlsbad, CA, USA) to make the storage solution. The storage solution was diluted to a proper concentration just before each experiment. A concentration of 200 μM (i.e., 3.6 g/L) ferrous myoglobin was used in this study. Only reduced myoglobin is cytotoxic, but only metmyoglobin was commercially available, so the myoglobin and ascorbic acid solutions were mixed to make the ferrous myoglobin medium as described in the literature [15]. The final concentrations of myoglobin and ascorbic acid were 200 μM and 2 mM, respectively. The ascorbic acid had reduced myoglobin to a ferrous status when the color changed from brown to reddish.

Human renal proximal tubular epithelial cells (HK-2) were grown in DMEM/F12 (1:1) medium with 10% v/v fetal bovine serum at 37°C in a humidified incubator with an atmosphere 5% CO₂. When the cells were 85% confluent, they were starved overnight (14 h) in serum-free medium to synchronize cell growth. After starvation, the following groups were evaluated: Con, HK-2 cells in normal media; Mb, HK-2 cells in ferrous myoglobin medium; Mb+NC, HK-2 cells in ferrous myoglobin medium transfected with vehicle; Mb+TTR, HK-2 cells in ferrous myoglobin medium transfected with TTR pcDNA-TTR.

Cell viability and injury

The HK-2 cells were cultured in 96-well plates. After synchronization for 16 h with low-serum medium, cells in the Con, Mb, Mb+NC and Mb+TTR groups were incubated for 24 h. The cells were then washed and assessed via the 3-(4, 5)-dimethylthiazolium(-z-y1)-3, 5-diphenyltetrazolium bromide (MTT) assay. The culture medium was transferred carefully to another test tube and centrifuged (1, 200 r/min 5 min), after which the supernatant lactose dehydrogenase (LDH) was measured.

Intracellular reactive oxygen species (ROS) assays

Cells were seeded onto 35-mm confocal dishes (with a cover glass) from the Con, Mb, Mb+NC and Mb+TTR groups (n = 3). After grouping, cells were cultured for 24 h followed by incubation with the total oxidative stress indicator chloromethyl derivative dichlorodihydrofluoresceindiacetate (CM-H₂DCFDA, Beyotime, Nanjing China, 5 μM) for 30 min in the dark at 37°C. After three washes with PBS, green fluorescence was visualized using a laser scanning confocal microscope at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Cellular immunofluorescence

HK-2 cells were seeded onto six-pore plates and covered by a cover glass that was pretreated with collagen I (Sigma-Aldrich, St. Louis, MO, USA). Each group was subjected to its own designated treatment regimen and the cells were cultured at 37°C for 24 h. The cells were fixed with 4% v/v paraformaldehyde for 5 minutes at room temperature followed by incubation at 4°C for 10 min. Then, 0.2% v/v Triton X-100 was added to permeabilize the cells for 5 min at room temperature and 1× casein was used to block the cells for 30 minutes at room temperature. Primary rabbit polyclonal antibodies specific for synaptopodin and nephrin were diluted 1:50 v/v and incubated with the cells (Santa Cruz Biotechnology) (ProSci Incorporated, San Diego, California, USA) overnight at 4°C. A secondary anti-rabbit antibody conjugated to Cy3 (Jackson Immuno Research Laboratories, Bar Harbor, ME, USA) (diluted 1:1000 v/v) was incubated with the cells away from light for one hour at room temperature. Finally, a fluorescent sealing liquid (ZSGB-BIO, Beijing, China) containing 4',6-diamidino-2-phenylindole (DAPI) was added, and confocal laser scanning microscopy (FluoView FV1000; Olympus America Inc., Center Valley, PA, USA) was used to determine the expression level and structural arrangement of TTR. Ten visual fields were observed at random in each culture pore.

Cell protein expression

The HK-2 cells were cultured in 6-well plates. After synchronization, cells were allocated to the Con, Mb, Mb+ NC and Mb+ TTR groups. At 24 h of incubation, the cellular protein was extracted. Then, TTR, cleaved caspase-3 and β -actin protein expression levels were determined by Western blot. Protein expression was measured by examining the relative intensity of bands. All antibodies were purchased from Abcam (Abcam, Cambridge, MA, USA).

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 17.0.2 software (IBM Corporation, Armonk, NY, USA). The results are presented as the mean values $\pm$ standard deviation (SD). Multiple comparisons of parametric data were performed using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls post hoc test. Student's t-test was used to compare differences between means. A P-value of < 0.05 was considered significant.

Results

Differential gene expression microarray analysis

We used glycerol-induced renal injury as a model of RM-induced AKI. The gene and expression data for three untreated mice were compared to the data for five mice with RM-induced AKI as we previously reported [9].

We used gene microarray analysis to detect differentially expressed genes between RM-induced AKI and normal murine renal tissues. Compared with the normal group, a total of 1709 genes were changed by more than 1.5-fold in the AKI group, of which, 659 genes were upregulated and 1050 were downregulated (Table 2).

Differential protein expression profiling with iTRAQ-MS

iTRAQ-MS was used to screen and identify differentially expressed proteins between the AKI group and the normal group. In total, 102 proteins were identified and quantified at the protein expression level. Among 102 proteins, 31 and 71 were up and downregulated, respectively (Table 2).

Table 2. Differentially expressed genes and proteins in rhabdomyolysis-induced AKI

Group	Number of significant differential genes			Number of significant differential proteins			Number of common differential genes and proteins
	Upper	Down	Total	Upper	Down	Total	
AKI/normal	659	1050	1709	31	71	102	17

Gene ontology analysis of the differentially expressed genes and proteins

Following careful analysis of the gene microarray and iTRAQ-MS results, we ultimately screened 17 common elements after multiple testing corrections. The regulatory directions of the 17 genes were in accordance with their proteins and thus became our key study objects (Fig. 1) (Table. 3).

Among the 17 genes and proteins, 10 were upregulated and 7 were downregulated according to the gene microarray (Table 4.). According to the iTRAQ-MS results, 6 were upregulated and 11 were downregulated (Table 4.). Among the 17 genes and proteins, 7 genes and proteins showed the same trend in the analysis. Afm, Cfi, Ttr, and Klf12 were downregulated while Psma5, Vcl, and Klf10 were upregulated.

Fig. 1. The numbers of differential genes detected by gene microarray and proteins by iTRAQ-MS. The red circles represent proteins identified by iTRAQ-MS, the blue circles represent genes identified by gene microarray, and the intersections between blue and red circles contain proteins and their corresponding genes.

Table 3. Information of the final selected 17 genes and their proteins

Gene ID	Gene	Description	Peptides	Gene express ratio(EXP/CON)	style (gene)	Protein express ratio(EXP/CON)	style (protein)
26442	Afm	afamin	13	0.3	down	0.72	down
269378	Cfi	complement component factor i	3	0.45	down	0.33	down
12630	Ttr	transthyretin	6	0.58	down	0.62	down
26446	Ahcy	adenosylhomocysteine hydrolase	1	0.58	down	1.23	up
22330	Ckb	creatine kinase, brain	3	0.58	down	1.67	up
15439	Pcolce	procollagen C-endopeptidase enhancer protein	2	0.59	down	1.29	up
16597	Klf12	Kruppel-like factor 12	9	0.54	down	0.57	down
22139	Psma5	proteasome (prosome, macropain) subunit, alpha type 5	4	2.05	up	1.26	up
20201	Vcl	vinculin	20	1.75	up	1.21	up
21847	Klf10	Kruppel-like factor 10	15	1.87	up	1.26	up
18542	S100a8	S100 calcium binding protein A8 (calgranulin A)	2	1.9	up	0.75	down
280662	Serpina3n	serine (or cysteine) peptidase inhibitor, clade A, member 3N	13	17.03	up	0.65	down
12709	Psmb3	proteasome (prosome, macropain) subunit, beta type 3	2	1.66	up	0.63	down
12224	Klf5	Kruppel-like factor 5	6	2.38	up	0.79	down
16691	Krt8	keratin 8	3	3	up	0.41	down
23849	Klf6	Kruppel-like factor 6	4	4.23	up	0.84	down
20716	Hp	haptoglobin	12	2.64	up	0.16	down

To obtain a broad assessment of the molecular functions, biological processes and cellular components of the 17 common elements, we performed gene ontology enrichment analysis using the DAVID v6.7 Functional Annotation Clustering tools (for all online suppl. material, see www.karger.com/doi/10.1159/000484028, Suppl. Table 1). The enriched pathways were analyzed using multiple online databases, including KEGG PATHWAY (Available online: <http://www.genome.jp/kegg>), BioCyc (Available online: <http://biocyc.org>) and GenMAPP (Available online: <http://www.genmapp.org>). (see online suppl. material, Suppl. Fig. 1). In the signaling pathway enrichment analysis, we found that the expression of Klf10 and Klf12, members of The Krüppel-like factor (Kfl) family, showed significant changes during RM-induced AKI.

The gene ontology analysis (GO) was classified into three functional groups: the molecular function group, the biological process group, and the cellular component group. In general, the main biological processes that were up- and downregulated in RM-induced AKI were cellular physiological processes and metabolic processes. For the molecular functions, the majority of the up- and down-regulated proteins clustered in protein binding. With regard to cellular components, up- and down-regulated proteins were mainly enriched intracellularly (Fig. 2).

Transthyretin selection

To perform an in-depth analysis of Afm, Cfi, Ttr, Psma5 and Vcl, we performed gene Pathway Network analysis for the 5 elements (Fig. 3). The Pathway Network analysis showed that TTR had a close relationship with electrochemical potential-driven transporter activity pathways. In

Table 4. Common differentially expressed genes and proteins in rhabdomyolysis-induced AKI

Group	Number of common differential genes			Number of common differential proteins		
	Upper	Down	Total	Upper	Down	Total
AKI/normal	10	7	17	6	11	17

Fig. 2. Gene ontology enrichment analyses for (a)biological process, (b)molecular function and (c)cellular component of the 17 differential genes and protein.

Fig. 3. Gene pathway network analysis for Afm, Cfi, Ttr, Psma5 and Vcl.

mitochondria, proton gradients are used to generate chemiosmotic potential, also known as proton motive force. The generation of ATP by oxidative phosphorylation leads to the production of various reactive oxygen species (ROS) in mitochondrial and submitochondrial particles. We suspected that TTR had a range of contact with ATP and ROS. Simultaneously, TTR can cause renal amyloidotic disease. The plasma TTR concentration is reduced in conditions involving inflammation and protein malnutrition [16]. Sousa et al. [17], showed that transthyretin (TTR) amyloid fibrils can have affinity for RAGE (receptor for advanced glycation end products) and subsequently induce NF-kappa-B activation and apoptosis. We found that TTR has a close relationship with kidney diseases. As such, we selected TTR as our key study object and investigated the mechanism of action of TTR in the kidney.

Use of an established RM-induced AKI animal model for verification

We established an RM-induced AKI mice model by glycerol injection to investigate the accuracy of the microarray results. We examined the levels of Afm, Cfi, Ttr, Psma5, Vcl, Klf10 and Klf12 using RT-PCR. Our results showed that, compared with the control group, the mRNA expression levels of Psma5, Vcl and Klf10 were all significantly increased. Furthermore, the mRNA expression levels of Afm, Cfi, Ttr and Klf12 were all significantly decreased (Fig. 4).

Our results showed that glycerol administration resulted in significant increases in the serum creatinine (Scr) and blood urea nitrogen (BUN) levels in the Gly group compared to the control group. We also investigated urinary Kim-1 levels as a biomarker of AKI. Urinary Kim-1 levels were significantly elevated in the RM-induced AKI group compared with the control group (Table 5). Control mice did not show any significant tubular damage. The rhabdomyolysis (RM) mice showed tubular necrosis, tubular dilation and cast formation (Fig. 5a and 5b).

Validation of downregulated TTR expression by immunohistochemistry and RT-PCR

To verify the gene microarray and iTRAQ-MS data, we conducted immunohistochemistry of TTR in RM-induced AKI renal tissues and normal renal tissues. Fig. 5c illustrated the

Table 5. Serum creatinine (Scr), blood urea nitrogen (BUN) and urinary kidney injury molecule-1 (Kim-1) concentrations, * $p < 0.05$ versus Control

	Control ($\bar{x} \pm \text{SEM}$)	Gly ($\bar{x} \pm \text{SEM}$)
Scr (mg/dl)	0.18 $\pm$ 0.04	1.14 $\pm$ 0.19*
BUN (mg/dl)	24.9 $\pm$ 3.94	110.7 $\pm$ 9.81*
Kim-1 (ng/ml)	0.22 $\pm$ 0.2	6.18 $\pm$ 1.4*

Fig. 4. The results showed that compared with the control group, the mRNA expression levels of Psma5, Vcl and Klf10 were significantly increased and the mRNA expression levels of Afm, Cfi, Ttr and Klf12 were significantly decreased. * $p < 0.05$ versus Control.

cellular localization of the TTR protein. The immunohistochemical results showed that the TTR protein can be detected in tubules. TTR was expressed in most of the tubular epithelial cells of mice in the normal group, but was significantly decreased in the AKI group (Fig. 5c). We also examined the levels of TTR using RT-PCR. Our results showed that compared with the control group, the mRNA expression levels of TTR were significantly decreased. (Fig. 5d)

Mitochondrial function

Rhabdomyolysis increased the production of ROS. Compared with the normal group, ROS production was significantly increased in the AKI group (Fig. 5e). This result indicated that mitochondrial function was impaired in the AKI group. Approximately 72 h after model induction, we also found that ATP, a marker of respiratory function in mitochondria, was markedly decreased, indicating that RM suppresses mitochondrial respiratory function (Fig. 5f).

Western blot analysis and confirmation of expression.

Western blot analysis of the AKI and normal renal tissue samples was performed to assess whether the TTR protein was differentially expressed. After incubation with the anti-TTR antibody, a distinct band at approximately 16 kDa was detected in each lane. Corresponding to the SDS gel analysis, Western Blot analysis also showed decreased expression of TTR in the AKI group. These alterations in TTR protein abundance between normal and AKI tissues were consistent with the gene microarray and iTRAQ-MS results. Apoptosis is an important phenomenon in RM-induced AKI. Caspase-3 acts as an “executioner” protein in the apoptotic pathway. We thus examined the protein expression of cleaved caspase-3 in the AKI and normal group. An upregulation of cleaved caspase-3 was observed in the AKI group compared to the control group (Fig. 5g and 5h).

Anti-apoptotic effect of TTR in HK-2 cells

We next investigated myoglobin-induced apoptosis in HK-2 cells *in vitro* to discover the apoptosis-related mechanisms of TTR proteins in RM-related AKI. To investigate whether TTR has an anti-apoptotic effect, we cultured HK-2 cells transfected with TTR pcDNA-TTR. Vehicle was transfected in the same way as the negative controls. pcDNA-TTR was transfected into HK-2 cells to overexpress the TTR gene. The HK-2 cells were incubated in normal media (Con), 200 μM ferrous myoglobin media (Mb), ferrous myoglobin plus vehicle (Mb+NC) and ferrous myoglobin plus pcDNA-TTR (Mb+TTR).

Immunofluorescence of TTR was applied to evaluate the effect of Mb and pcDNA-TTR transfection on HK-2 cells. The expression of TTR immunofluorescence was significantly

Fig. 5. Renal function, mitochondrial function and TTR expression in rhabdomyolysis (RM)-induced acute kidney injury (AKI). (a) Periodic acid-Schiff-stained sections of mouse kidneys (200×) in normal (Con) and RM-induced kidney section. (b) Acute tubular necrosis (ATN) scores at 72 h after rhabdomyolysis. (c) Expression of TTR in kidney tissues stained by immunohistochemistry. (d) Downregulated of TTR mRNA expression in RM-induced AKI. (e and f) Reactive oxygen species (ROS) relative level in renal tubules and ATP relative level in renal tubular mitochondria. (g) Western blot for TTR and cleaved caspase-3 in AKI and normal renal tissues. (h) Relative quantification of the TTR and cleaved caspase-3 expression in (g), normalized to actin. * $p < 0.05$ versus Con group.

downregulated after incubation with ferrous myoglobin or vehicle. However, the expression of TTR immunofluorescence was upregulated by pcDNA-TTR transfection (Fig. 6a).

The MTT assay showed that the cell viability of HK-2 cells incubated with ferrous myoglobin was lower than that of the control ($p < 0.05$). Vehicle had no effect on the viability of HK-2 cells. Transfection of pcDNA-TTR significantly increased the viability of HK-2 cells ($p < 0.05$) (Fig. 6b).

Supernatant LDH release assays indicated that the LDH release of HK-2 cells incubated with ferrous myoglobin was higher than that of control cells ($p < 0.05$). Supernatant LDH release from HK-2 cells in the Mb+TTR group was lower than in the Mb+NC group ($p < 0.05$) (Fig. 6c). These results provide evidence that TTR may have an anti-apoptotic effect.

To better understand the molecular mechanisms underlying the anti-apoptotic effects of TTR, we investigated the effects of TTR on ROS generation in HK-2 cells since previous studies have provided strong evidence that TTR has a close relationship with apoptosis and the accumulation of reactive oxygen species (ROS) [18]. Consistent with the results from previous studies, ROS levels were increased when the HK-2 cells were incubated with the Mb and vehicle for 24 h. Transfection of pcDNA-TTR significantly decreased ROS levels compared with vehicle (Fig. 6d and 6e).

In another experiment, HK-2 cells were incubated for 24 h in the Con, Mb, Mb+NC and Mb+TTR groups. The total protein of the cells was extracted, and apoptosis-related protein expression was measured by Western blot. We found that myoglobin incubation increased cleaved caspase-3 and decreased TTR, while transfection of pcDNA-TTR significantly decreased cleaved caspase-3 and increased TTR compared to vehicle. (Fig. 6f and 6g). These

Fig. 6. Effect of TTR over expression on the cell viability, cell injury, reactive oxygen species (ROS), mitochondria function and protein expressions of myoglobin-induced apoptosis in HK-2 cells. (a) Immunofluorescence staining of HK-2 cells showed the expression of TTR. TTR expression was detected by CY3-labeled antibody; Nuclei were counterstained with DAPI. (b) The MTT assay showed the cell viability of HK-2 cells. (c) Supernatant LDH release tests indicated cell injury of HK-2. (d) Effect of different Mb or TTR treatment on intracellular oxidative stress by oxidant-sensing fluorescent probe DCFDA. (e) Reactive oxygen species (ROS) relative level in renal tubules. (f) Densitometric quantifications of band intensities from western blot for TTR and cleaved caspase-3/β-actin in HK-2 cells. (g) Representative tissue TTR and cleaved caspase-3 protein expression. The relative density of cleaved caspase-3 to β-actin was compared. (* $P < 0.05$ vs. Con group; # $P < 0.05$ vs. Mb group).

results suggest that TTR plays a cytoprotective role by reducing cell apoptosis and reducing cellular oxidative stress.

Discussion

No specific therapy is currently available for human AKI, a clinical entity characterized by increasing incidence and high morbidity and mortality. While technological advances have enabled decreases in many diseases, rhabdomyolysis remains one of the leading causes of AKI, developing after trauma, drug toxicity, infections, burns, and physical exertion. Research on the mechanism of rhabdomyolysis-related AKI is therefore ongoing [19, 20]. An animal model using an intramuscular glycerol injection with consequent myoglobinuria is closely related to the human syndrome of rhabdomyolysis [21].

By integrating proteomics with gene microarray, we conducted this research to study the mechanism of rhabdomyolysis in AKI. Isobaric tagging with related and absolute quantitation (iTRAQ) labeling mass spectrometry (MS) was applied to screen and identify differentially expressed proteins between RM-induced and normal renal tissues. Affymetrix U133 plus 2.0 microarrays were used to perform gene microarray analysis. Gene microarray integrated with iTRAQ-MS of RM-induced and normal renal tissues identified over 1700 expressed genes and 100 expressed proteins, including 17 common elements. Gene ontology enrichment analysis was used to obtain a broad assessment of the molecular functions, biological processes and cellular components of the 17 common elements. In the 17 genes and proteins, we focused on 7 genes and proteins with the same trend: *Afm*, *Cfi*, *Ttr* and *Klf12* were downregulated while *Psma5*, *Vcl* and *Klf10* were upregulated. We also performed gene Pathway Network analysis for the 7 common elements. Interestingly, we found that TTR was related to the electrochemical potential-driven transporter activity pathway. An electrochemical gradient is a gradient of electrochemical potential, usually for an ion that can move across a membrane. ATP synthesis via F1/Fo-like ATP synthases is a universal energy conversion process in all living cells. An essential step of this process is the utilization of free energy from the transmembrane electrochemical potential of cations. In the mitochondria, generation of ATP by oxidative phosphorylation leads to the production of various reactive oxygen species (ROS) [22]. At the same time, abnormal TTR can cause renal amyloidotic disease. We suspected that TTR may be related to mitochondria, ATP, ROS and apoptosis. As such, we selected TTR as our key study object and investigated the association between TTR and an anti-apoptotic effect.

TTR is one of the most abundant circulating proteins. It belongs to a subset of 25 proteins with a predisposition to misfold. It is synthesized by the liver (90%), retina, pancreas, and choroid plexus as a 55 kDa tetramer with four identical 127-residue β -sheet-rich subunits and a 20-residue signal peptide with no specific physiologic properties [23]. TTR mRNA has been found in kidney cells [24]. TTR binds to thyroxine and retinol-binding protein in the blood and cerebrospinal fluid and transports vitamin A without losing retinol-binding protein molecules in the kidney [25].

To further verify the gene microarray and iTRAQ-MS results, immunohistochemical staining was used to confirm the differential expression of the TTR protein between RM-induced AKI and normal murine renal tissues. A hypertonic glycerol injection in mice is an established method used to study the pathogenesis of RM-induced AKI. The immunohistochemical results showed that the TTR protein can be detected in tubules. TTR was expressed in most of the tubular epithelial cells of the normal group, but was significantly decreased in the AKI group.

The generation of ATP by oxidative phosphorylation leads to the production of various reactive oxygen species (ROS) in the mitochondria and submitochondrial particles. The formation of ROS as a mitochondrial waste product will eventually lead to cytotoxicity and cell death. Because of their role in metabolism, mitochondria are very susceptible to ROS damage. Damaged mitochondria result in the depletion of ATP and the release of cytochrome c, which leads to the activation of caspases and the onset of apoptosis. Mitochondrial damage is not caused solely by oxidative stress or disease processes; normal mitochondria will eventually accumulate hallmarks of oxidative damage over time, which can be deleterious to mitochondria as well as the cell [26, 27]. These defective mitochondria can further deplete ATP from the cell, increase production of ROS, and release pro-apoptotic proteins such as caspases. Apoptosis is an important phenomenon in RM-induced AKI. Caspase-3 acts as an "executioner" protein in the apoptotic pathway [28]. We assessed ROS, ATP and apoptosis-related cleaved caspase-3 in normal mice and the RM-induced AKI group. Accompanied by TTR downregulation, our results showed that ROS production was significantly increased, ATP was markedly decreased and cleaved caspase-3 was upregulated in the AKI group. These findings indicated that RM leads to the production of ROS, suppressed mitochondrial respiratory function and the induction of apoptosis.

To elucidate the details of TTR function, we investigated the apoptosis of HK-2 cells *in vitro*. We induced apoptosis in HK-2 cells by adding ferrous myoglobin to replicate rhabdomyolysis-induced AKI. To investigate whether TTR has an anti-apoptotic effect, we constructed pcDNA-TTR to overexpress the TTR gene. Our results showed that decreased TTR was accompanied by increased ROS generation, whereas overexpression of TTR reduced the total ROS levels in HK-2 cells. Transfection of pcDNA-TTR significantly increased the viability of HK-2 cells. Previous studies have demonstrated that intracellular ROS causes damage to cellular DNA, leading to apoptosis [29, 30]. Myoglobin incubation increased cleaved caspase-3 and decreased TTR, while transfection of pcDNA-TTR significantly decreased cleaved caspase-3 and increased TTR. Thus, it is conceivable that TTR exerts anti-apoptotic effects by reducing ROS, which subsequently reduces apoptosis. Our results showed that a reduction in TTR could increase the generation of ROS and the occurrence of apoptosis in HK-2 cells, consistent with previous reports that TTR oligomers exert an apoptotic response [31, 32].

During our proteomics with genomics analysis, we had some interesting and novel discoveries. Our research indicated that TTR may exert an anti-apoptotic effect on RM-induced AKI. Zang X showed that TTR shifts the balance of Bcl2 proteins toward anti-apoptotic proteins resulting in reduced cell death. These results resemble those observed in a kidney cell line, where NGAL (neutrophil gelatinase-associated lipocain) was able to reduce apoptosis in a hypoxia-reperfusion model by regulating Bax and Bcl2 levels in a megalin-dependent manner [33]. We also found that megalin was downregulated in our gene analysis. Megalin, a low density lipoprotein-related protein 2 also known as LRP2, is a protein which is encoded by the *LRP2* gene in humans. TTR binds megalin, RAGE9 (receptor for advanced glycation end products) and IGF-IR (insulin-like growth factor 1 receptor). Megalin has been mainly studied in the kidney. Megalin activates intracellular pathways, such as Erk1/2 and Akt, leading to the activation of the transcription factor CREB. The gene microarray showed that megalin was also downregulated. Gomes J R et al. showed that the interaction of TTR with megalin activates MAP kinase, ERK, Akt and Src, ultimately leading to the upregulation of the CREB transcription factor, as seen for other LRP ligands [34]. TTR is thus a novel megalin ligand with potential importance in T4 transepithelial transport, reinforcing the concept that megalin is a general endocytic receptor for protein in the proximal tubule with a multifaceted role in retaining and capturing vital substances from the tubular fluid after glomerular filtration [35]. TTR binding of megalin may activate MAP kinase, ERK, Akt and Src, indicating that TTR might play an important role in the pathogenesis of RM-induced AKI. Interestingly, our research showed that the expression of Klf10 and Klf12, members of The Krüppel-like factor (Klf) family, exhibited significant changes in the process of RM-induced AKI. The Krüppel-like factor family of transcription factors regulates diverse biological processes that include proliferation, differentiation, growth, development, survival, and responses to external stress. Seventeen mammalian KLFs have been identified, and numerous studies have been published that describe their basic biology and contribution to human diseases. Klf5, Klf6, Klf10, Klf12, Klf15, and Klf16 are expressed in the developing kidney and adult kidney [36]. A further more comprehensive study is needed to describe the role of KLFs in RM-induced AKI.

Our study integrated high-throughput proteomics with genomics and identified TTR as an important protein involved in RM-induced AKI. Our results suggested that TTR was downregulated, while caspase-3, an apoptosis “executioner” protein, was upregulated in RM-induced AKI. Further *in vitro* experiments showed that a reduction in TTR could increase the generation of ROS and induce apoptosis. Our findings provide a platform for the further characterization of genes implicated in RM-induced AKI and may provide new insights into the mechanisms of RM-induced AKI.

Acknowledgements

This research was supported by grants from the National Natural Science Foundation of China (No. 81470949) and the Major State Basic Research Development Program of China (NO. 2013CB530800 and NO. 2014CBA02005)

Disclosure Statement

No conflicts of interest.

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