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Serum Proteomic Profiling Analysis of Rats Chronically Exposed to Arsenic

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: Arsenic (As) is an environmental contaminant, and As pollution in water and soil is a public health issue worldwide. As exposure is associated with the incidence of many disorders, such as arteriosclerosis, diabetes, neurodegenerative diseases, and renal dysfunction. However, the mechanism of As toxicity remains unclear.

Material/Methods: We investigated the changes in serum protein profiles of rats chronically exposed to As. Twenty healthy rats were randomly divided into 4 groups, and sodium arsenite of varying final concentrations (0, 2, 10, and 50 mg/L, respectively) was add into the drinking water for each group. The administration lasted for 3 months. Two proteomic strategies, isobaric tags for relative and absolute quantitation (iTRAQ), and 2-dimensional gel electrophoresis (2-DE), were employed to screen the differential serum proteins between control and arsenite exposure groups.

Results: We identified a total of 27 differentially-expressed proteins, among which 9 proteins were significantly upregulated and 18 were downregulated by As exposure. Many of the differentially-expressed proteins were related to fat digestion and absorption, including 5 apolipoproteins, which indicated lipid metabolism may be the most affected by As exposure.

Conclusions: This study revealed the influence of As on lipid metabolism, suggesting an increased potential risk of relevant diseases in subjects chronically exposed to As.

MeSH Keywords: Arsenic • Electrophoresis, Gel, Two-Dimensional • Proteomics • Rats, Inbred Strains

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Background

Arsenic (As) pollution remains a serious public health concern worldwide. More than 100 million people are chronically exposed to elevated levels (over) of As in their drinking water, and 10 µg/L is the upper limit recommended by World Health Organization (WHO) in 2011 [1]. Many countries have regions where drinking water is contaminated by As, such as Bangladesh, West Bengal of India, the United States, Argentina, Mexico, and China [2]. According to an estimate by Sun et al., there are about 6 million people in China exposed to As with the concentration over the WHO limit; of these, around 1.9 million are exposed to a concentration exceeding 50 µg/L [3].

Chronic As exposure has been implicated in several diseases, including dermatosis, diabetes mellitus, hypertension and cardiovascular disease, abnormal porphyrin metabolism, hyperpigmentation, and hyperkeratosis [4–6]. Significant hearing loss [7], adverse impacts on intelligence, learning disability, and memory decline [6] have been recorded in children who live in As-polluted areas. As is considered a group 1 carcinogen by the International Agency for Research on Cancer, and prolonged exposure is associated with skin, bladder, liver, and lung cancer [8]. However, the mechanisms underlying As-induced disease remains unclear, though several mechanisms have been proposed, including oxidative stress.

As metabolism is a complex process, with 5 As species identified in urine. These species include inorganic arsenics, monomethyl arsenous acid, monomethyl arsonic acid, dimethyl arsenous acid, and dimethylarsinic acid [9]. The efficiency of methylation of these species may be a critical factor following inorganic As exposure, because varying proportions of the As species are differentially associated with As-induced outcomes such as atherosclerosis, hypertension, cancer, and nervous system diseases [10]. Therefore, differences in the efficiency of As methylation may play a role in the incidence of As metabolites-associated disease.

Most evidence about As toxicity has originated from studies on high As exposure, but more evidence is needed on the health effects of chronic low As exposure, and few protein biomarkers are effective in early diagnosis of As-induced damage [3]. Therefore, we performed the present *in vivo* study of As toxicity at varying concentrations, hoping to find early changes induced by arseniasis in serum proteins, and to explore the mechanisms underlying the toxicity of As exposure.

For analyses, conventional biochemical and immunological assays are not suitable for detecting low levels of proteins in serum [11]. iTRAQ is a well-established *in vitro* isotope-labeling technique developed by Applied Biosystems, Inc. in 2004, which is usually combined with mass spectrometry online [12–14]. Coupled with HPLC-tandem mass spectrometry, iTRAQ can simultaneously identify thousands of proteins assessed by 8-plex measurement to provide comprehensive information [15,16]. In recent years, iTRAQ-based quantitative methods have been widely used in proteomic studies of neurodegenerative diseases [17], squamous cell lung cancer [18], gastrointestinal tract cancer [19], and breast cancer [20]. Two-dimensional electrophoresis (2-DE) is a canonical proteomic technique that visualizes differentially-expressed proteins and enables detection of potential post-translational modifications, such as phosphorylation and glycosylation [21].

The objective of the present study was to apply 2-DE and iTRAQ-based proteomic strategies to explore differentially-expressed serum proteins in rats chronically exposed to As. With varying levels of As exposure in drinking water (0 mg/L, 2 mg/L, 10 mg/L and 50 mg/L), lipid metabolism and proteasome were identified as important pathways involved in the toxicity. These results provide evidence of the early biological effects of As exposure, indicating that the impacts of varying levels of As exposure on serum proteomic profiling.

Material and Methods

Animals and arsenic exposure *in vivo*

Twenty adult Sprague-Dawley rats weighing between 170 and 200 g were purchased. Rats were obtained from the Medical Laboratory Animal Center (Guangdong, China). All rats were maintained in an environment at 23±2°C with 50±5% humidity and a 12-h light-dark cycle. The rats were randomly divided into 4 groups (5 rats in each). The rats were exposed to As with varying concentrations in the drinking water for 3 months at 2 mg/L, 10 mg/L, 50 mg/L, respectively. The control group was not exposed to sodium arsenite. All rats were fed a normal diet. As a previous study by Mazumder et al. [22] showed that rodents might be less susceptible than humans to arsenic toxicity because of faster arsenic metabolism and excretion, we selected an arsenic concentration higher than that found in the environment. These concentrations correspond to around 1.6 mg/L in humans according to the body surface area normalization method [23], which is representative of levels found in affected areas [24]. The rats were fasted overnight, and sacrificed under anesthesia with 10% chloral hydrate after the exposure. Blood samples (4 mL from each individual) were collected from the carotid artery into 5% vol heparin tubes (Venoject II, Terumo, Tokyo, Japan) and immediately centrifuged at 3000 g for 10 min at 4°C. The serum was separated and stored at –80°C until analysis. All animal experiments were approved by the Ethics Committee of the Xinjiang Uygur Autonomous Region Center for Disease Control and Prevention.

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Serum sample preparation

Equal amounts of serum from the 5 individuals in each group were combined. A ProteoExtract™ Albumin/IgG Removal Kit (Merck, Germany) was used to remove the high-abundance proteins in serum sample according to the manufacturer's instructions, and serum pools were then desalted with an ultrafiltration filter (Sartorius, Germany). Finally, the total protein samples were quantified with BCA (bicinchoninic acid) assay.

Arsenic level measurement

The levels of total arsenic in urine were determined by hydride generation atomic fluorescence spectrometry (HG-AFS), as described previously [25].

High-performance liquid chromatography (HPLC) and mass spectrometry analysis

Each sample was subjected to trypsin digestion and iTRAQ labeling. Subsequently, the digested samples were separated with a capillary HPLC system. The Q-Exactive mass spectrometer from Thermo Finnigan was used to perform data acquisition.

Samples were separated by Easy nLC system with EASY column (Thermo scientific, 10 cm×75 μm, 3 μm C18). The column temperature was set at 25°C and injection volume was 10 μL. The gradient elution of mobile phase was at a flow rate of 250 nL/min. The mobile phase consisted of 0.1% formic acid in water (mobile phase A) and 84% acetonitrile plus 0.1% formic acid in water (mobile phase B). The gradient elution procedure was as follows: phase B started from 0%, and then increased linearly to 35% at 50 min, and further increased to 100% at 55 min, then was held for 5 min. During the whole analysis, the samples were in an automatic 4°C injector. The samples were analyzed randomly. QC samples were inserted into the sample queue to monitor and evaluated the stability of the equipment and the reliability of the raw data.

MS analysis was performed using a Q-Exactive mass spectrometer (Thermo Finnigan) with a nano ESI source and an orbitrap analyzer, operating in positive ion modes. The mass spectrometer recorded ions with a range of 300–1800 m/z. The dynamic exclusion duration was 40.0 s. Survey scans were acquired at a resolution of 70 000 at m/z 200, and the resolution for the HCD spectra was set to 17 500 at m/z 200. The normalized collision energy was 30 eV, and the underfill ratio was defined as 0.1%. QC samples were checked at an interval of 7 samples to ensure stability during the whole sequence.

Data processing

Raw MS/MS spectra data were analyzed using Mascot (version 2.2, Matrix Science, London, U.K.). The search engine was set up to search the UniProt_rat database. Scaffold Q+ (version Scaffold_4.4.5, Proteome Software, Inc., Portland, OR, United States) was used to quantify the iTRAQ peptide. A threshold of±1.2-fold change with p value < 0.05 was considered as significant changes. Statistical procedure was performed with the *t* test. Gene Ontology (GO) annotation, including biological processes (BP), cellular components (CC), and molecular functions (MF), of the identified proteins was implemented using BLAST2TO (version 2.5), and pathway enrichment analysis of the differentially-expressed proteins was by Kyoto Encyclopedia of Genes and Genomes (KEGG) automatic annotation server.

Two-dimensional gel electrophoresis

For each gel, 100 μg of the protein sample was loaded. Isoelectric focusing was performed at 4°C at 8000 V for 10 h [26]. Subsequently, the equilibrated strip with proteins was transferred to the top of a polyacrylamide gel. The second dimension was conducted in 12% SDS-PAGE with running buffer (0.025 M Tris-base, 0.19 M glycine, 0.1% SDS). The protein spots were visualized with silver staining.

Gel analysis

The stained gels were scanned with a BearPaw 2448TA Pro scanner (Mustek, Taiwan, China). Relative quantification of protein expression in the samples was compared using PDQuest 2-D Analysis Software version 8.0.1 (Bio-Rad, USA). The differential spots were picked according to a 1.2-fold increase or decrease in relative intensity.

Statistical analysis

SPSS 20.0 statistical software (SPSS, Chicago, IL, USA) was used for data analysis. The indexes were analyzed by one-way ANOVA. Differences were set as significant at $P<0.05$.

Results

Urinary arsenic level of the rats

The levels of arsenic in urine at the end of the treatment period were significantly different between every 2 groups. The levels of arsenic in urine increased gradually as the doses of arsenic exposure increased (Figure 1).

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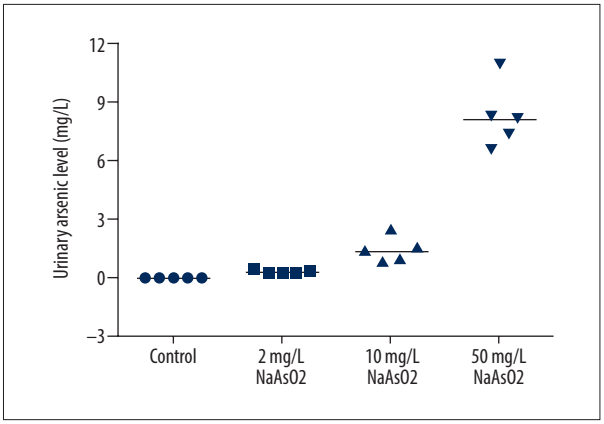

Figure 1. Urinary arsenic level among the groups, expressed as median. The levels of arsenic in urine increased gradually as the doses of arsenic exposure increased.

Spectrum and GO analysis of the identified proteins of the rat sera

A total of 4358 peptides in the serum samples were detected, which covers 628 proteins; of these, 178 differentially-expressed proteins (fold-change >1.2 or <0.83 in comparison with the control group, $p<0.05$) were found in all groups with quantitative information and were included in the next bioinformatic analysis. The 178 proteins were annotated according to their biological process, cellular component, and molecular function by BLAST2TO (Figure 2). Biological processes analysis showed that these proteins were mainly involved in biological regulation, cellular process, response to stimulus, single-organism process, and metabolic process. Cellular component analysis showed that most of the differential proteins were located in the extracellular region, membrane, and organelle. Molecular function analysis revealed that a large proportion of these proteins played a role in protein binding, catalytic activity, transporter activity, and molecular transducer activity.

Pathway enrichment analysis among the 178 significantly changed proteins by As exposure showed that 7 proteins were consistently upregulated in the low-, medium-, and high-As exposure groups, and 10 proteins were downregulated in all As exposure groups. Details of the consistent proteins in As exposure are listed in Table 1. One of the 17 differentially-expressed proteins was uncharacterized, which was excluded for the following pathway annotation. Pathway annotation by KEGG analysis demonstrated that these proteins are mainly involved in proteasome function (Pdzrn4, Psmb8), protein transportation (Sil1), PPAR (Peroxisome Proliferators-Activated Receptors) signaling pathway (Apoc1), endocytosis (RGD1308428), PI3K-Akt signaling pathway (Col1a1, Tsc1), and carbon metabolism (Sirt6) (Figure 3). The above pathways might be affected by arsenite treatment and may be involved in the mechanisms of As toxicity.

2-DE analysis and differential spots identification in rat sera

In this study, 2-DE was applied as a complementary strategy to investigate the serum protein profiling. The 2-DE gel images of the control group, low-As-exposed group, medium-As-exposed group, and high-As-exposed group are shown in Figure 4.

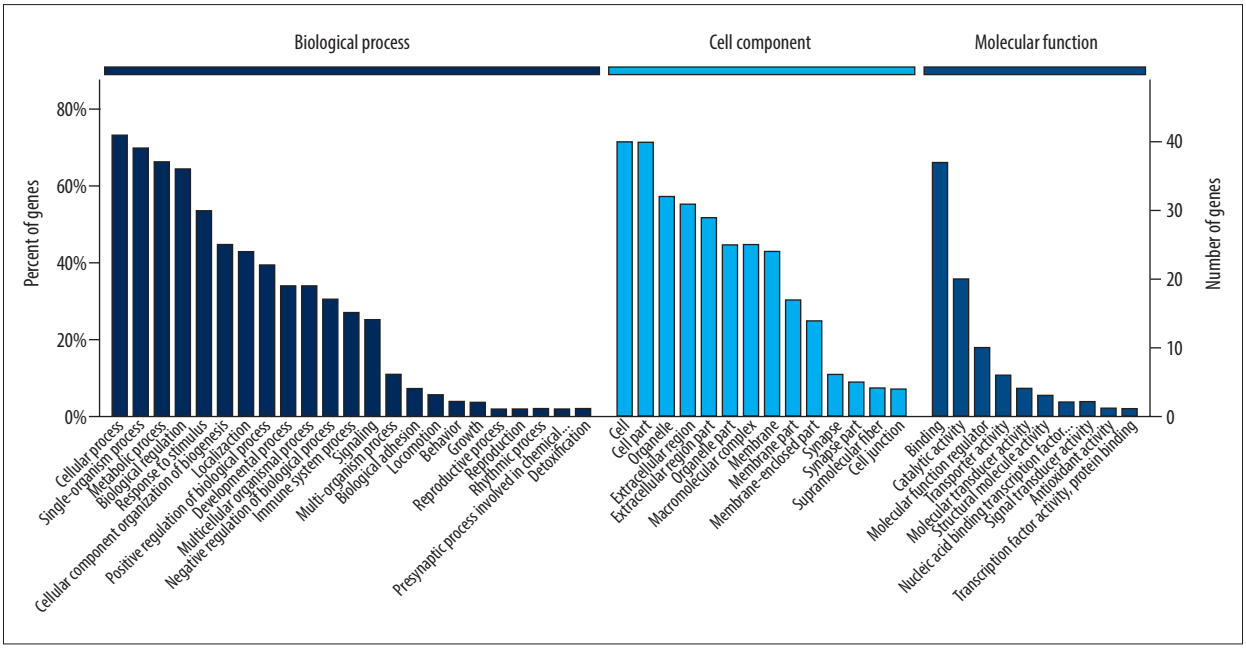

Figure 2. GO classification of the identified proteins of rat serum by iTRAQ: biological process, cellular component, and molecular function.

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Table 1. The list of serum differentially expressed protein through iTRAQ in rats induced by chronic arsenic exposure.

Accession	Protein	Gene name	Coverage	No. of unique peptides	MW [kDa]	Calc. pI	Ratio B/A	Ratio C/A	Ratio D/A
D3ZE08	Uncharacterized protein	ENSRNOG 00000045599	5.30	1	14.6	9.41	1.501	1.724	1.414
D3ZNV5	PDZ domain-containing RING finger 4	Pdzrn4	2.97	1	88.6	5.30	1.317	1.362	1.269
Q5FVP9	Cfh protein	LOC100361907	10.69	1	45.3	7.52	1.437	1.503	1.402
Q6P6S4	Nucleotide exchange factor SIL1	Sil1	2.15	1	52.3	5.14	1.939	1.726	1.840
M0R557	Cardiomyopathy-associated 5	Cmya5	0.56	1	433.3	4.75	1.710	1.322	1.512
P19939	Apolipoprotein C-I	Apoc1	38.64	5	9.9	9.09	1.287	1.432	1.620
A0A1W2Q628	Centrosomal protein 350	Cep350	0.83	1	190.0	5.35	1.653	2.200	4.707
P46462	Transitional endoplasmic reticulum ATPase	Vcp	4.22	2	89.3	5.26	0.794	0.714	0.615
P02454	Collagen alpha-1(I) chain	Col1a1	0.62	1	137.9	5.92	0.592	0.523	0.486
P69897	Tubulin beta-5 chain	Tubb5	30.63	2	49.6	4.89	0.652	0.571	0.534
A0A023IKI3	Proteasome subunit beta type	Psmb8	4.35	1	30.6	7.78	0.663	0.741	0.724
F1M037	Protein RGD1308428	Snx1	1.23	1	115.6	7.34	0.769	0.633	0.546
Q4FZY2	Protein Sirt6	Sirt6	3.03	1	36.3	8.44	0.773	0.593	0.524
D4A6Q6	Protein Ino80	Ino80	1.28	1	176.5	9.33	0.809	0.534	0.545
Q63369	Nuclear factor NF-kappa-B p105 subunit	Nfkb1	1.34	1	56.5	4.82	0.709	0.253	0.267
Q9Z136	Hamartin	Tsc1	1.29	1	128.9	6.32	0.442	0.669	0.711
M0R757	Elongation factor 1-alpha	Eef1a1	1.95	1	50.1	9.01	0.548	0.445	0.257

A – control group; B – low-As exposed group; C – medium-As exposed group; D – high-As exposed group.

After analyzing the images, 12 differential spots which were consistently up- or downregulated (fold-change >1.2 or <0.8) in all As-exposed groups were detected. The isoelectric point and molecular weight of the 12 protein spots were estimated according to the pH gradient of the IPG strip and protein markers, respectively. Identification of the proteins was by protein sequence alignment with the database SWISS-2DPAGE. Of these 12 protein spots, 10 were identified with confidence, as listed in Table 2.

The image analysis revealed that 2 of the differentially-expressed proteins were upregulated and 8 were downregulated. Functional analysis revealed that 4 out of the 12 differentially-expressed proteins are associated with fat digestion and absorption, which indicated lipid metabolism may be the pathway most affected by As exposure.

Discussion

Many epidemiological studies have shown associations between As exposure and a variety of human diseases, but the underlying mechanisms remain unclear [27]. There have been several mechanisms hypothesized regarding As carcinogenicity, including genotoxicity, altered DNA repair and DNA methylation, and oxidative stress [28]. In some non-cancer-related health

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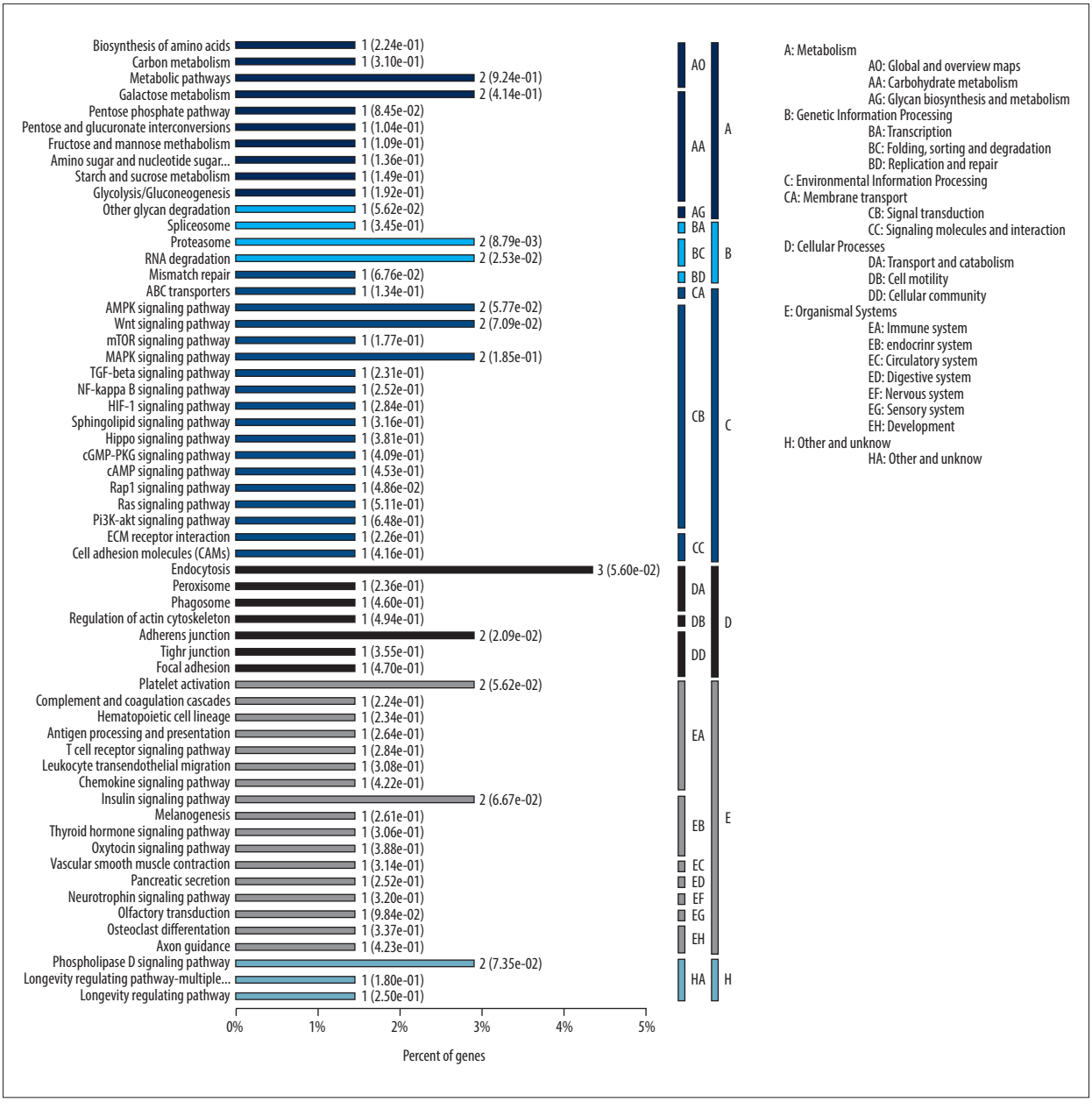

Figure 3. KEGG analysis of the identified proteins of rat serum by iTRAQ.

issues, As exposure also increased the incidence through increased oxidative stress, decreased expression of PPAR-γ, and inhibition of PDK-1 (Phosphoinositide Dependent Kinase-1) [28].

Serum is an easily accessible sample for screening of protein biomarkers, and it also reflects the overall health condition [29]. The proteomic strategy has the advantages of high throughout and low detection limit compared with conventional techniques, which facilitate to discovery of novel disease-related proteins [3].

In the present study, a total of 27 differentially-expressed serum proteins were identified by either iTRAQ or 2-DE, of which 9 were upregulated and 18 downregulated by As exposure. According to the literature, 6 differentially-expressed proteins – collagen alpha-1(I) chain, Proteasome subunit beta type, Nuclear factor NF-kappa-B p105 subunit, Apolipoprotein A-I, Apolipoprotein A-IV, and Apolipoprotein E – were reported to be associated with As exposure [30–35], while the others were not. Heat map generation and hierarchical clustering were performed on all samples and showed that the expression profiles of genes associated with lipid metabolism and proteasome pathway were clearly distinguishable (Figure 5).

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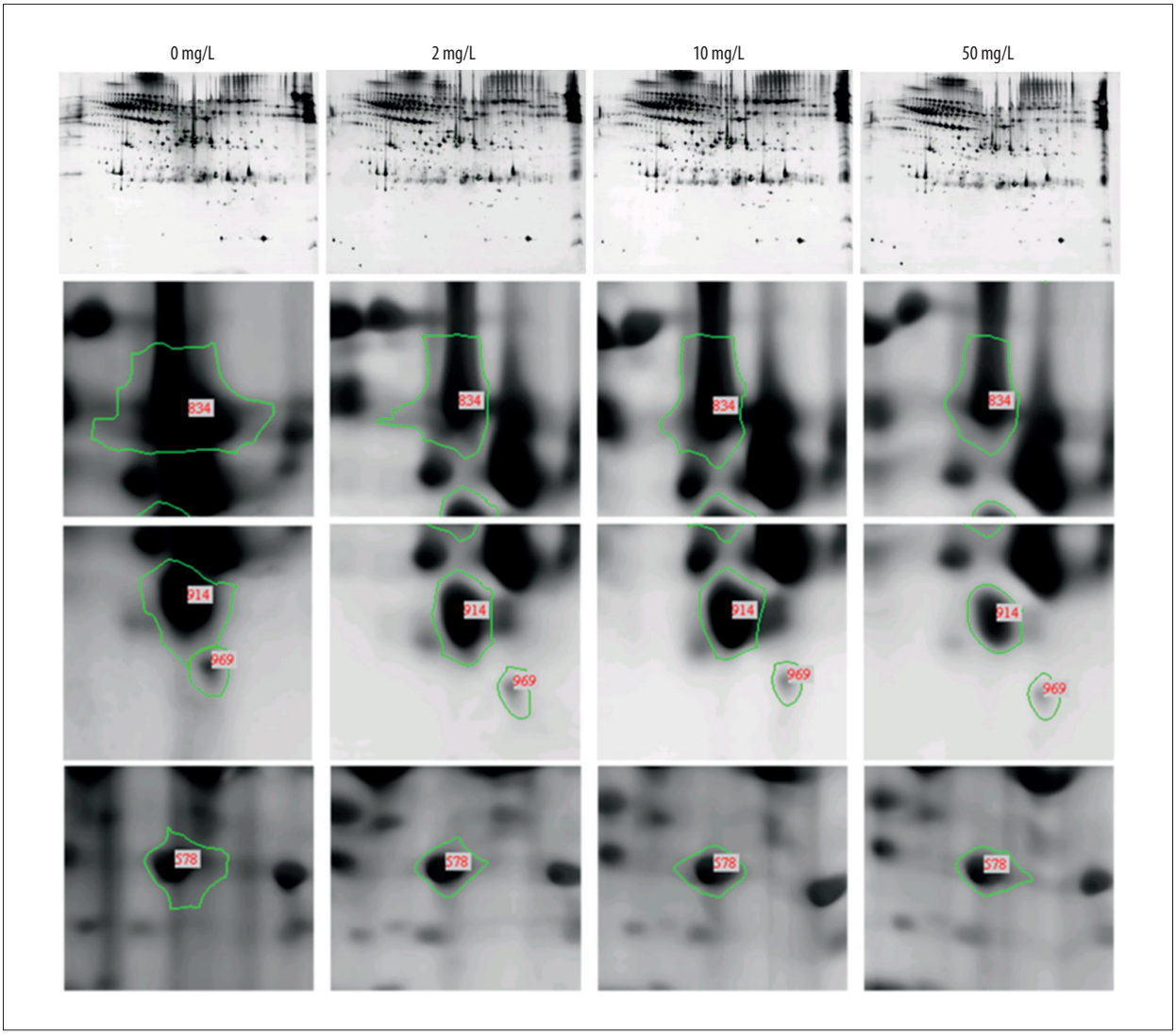

Figure 4. Representative images of two-dimensional gel electrophoresis of serum from the control group, low-As-exposed group, medium-As-exposed group, and high-As-exposed group, as indicated. We resolved 100 µg of serum proteins on pH 3–10 gradient in the first dimension and separated on 12% SDS-polyacrylamide gels in the second dimension, with the conditions 3 W/gel (920 V h) as described in Materials and Methods. The proteins chosen for analysis are marked by numbers.

2-DE is a classic proteomic approach, but it is usually limited due to its disadvantages in separating the proteins with extreme isoelectric point, molecular weight, or hydrophobicity, and the sensitivity also makes it unsuitable for use with low-abundance proteins; however, it can directly display the observed molecular weight, isoelectric point, relative abundance, and some potential post-translational modifications. iTRAQ is a quantitative proteomic approach with much wider application for various samples. Due to advances in modern mass spectrometry, iTRAQ has a better sensitivity and accuracy to acquire the quantitative information on low-abundance proteins. From the differentially-expressed proteins, we found many more hydrophobic and low-abundance proteins by use of iTRAQ than with use of 2-DE, and there were several apolipoproteins in the list of 2-DE, which verified the bias between the 2 approaches.

We found that the expression of several proteins was dependent on the As dose, such as Hamartin and Centrosomal protein 350. There are 4 possible interpretations of this effect. First, As could be an agent inducing the DNA CpG methylation upstream of the target genes [36], by which the gene expression depends on the dose of As. Second, the underlying mechanism of As toxicity was thought to be oxidative stress [28], which could lead the target proteins to form misfolding or improper modifications, and then the target proteins were recognized by the ubiquitin system, and finally they were degraded by proteasomes [37]. Thus, the level of target proteins is associated

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Table 2. The list of serum differentially expressed protein through 2-DE in rats induced by chronic arsenic exposure.

Accession	Protein	Gene name	Calc. MW [kDa]	Calc. pI	Ratio B/A	Ratio C/A	Ratio D/A
P04639	Apolipoprotein A-I	Apoa1	30.0	5.52	0.341	0.444	0.173
F7FF45	nuclear mitotic apparatus protein 1	Numa1	234.8	5.58	1.068	0.971	0.686
P02651	Apolipoprotein A-IV	Apoa4	44.4	5.12	0.7	0.418	0.461
Q5M7T5	Serine (or cysteine) peptidase inhibitor, clade C (antithrombin), member 1	Serpinc1	52.2	6.18	0.702	0.457	0.603
A0A0G2JWG6	Golgin subfamily B member 1 isoform X4	Golgb1	364.0	5.02	0.744	0.684	0.738
Q5U2Y3	Preprohaptoglobin	Mpp7	30.1	7.16	0.682	0.696	0.359
P01026	Complement component C3	C3	31.8	5.73	0.538	0.541	0.376
P02774	Group specific component, isoform CRA_b	GC	53.5	5.65	0.703	0.535	0.595
P02650	Apolipoprotein E, isoform CRA_c	Apoe	27.4	7.93	1.235	1.18	1.24
G3V6A8	Golgi autoantigen, golgin subfamily b, macrogolgin 1, isoform CRA_a	Golgb1	250.9	4.96	2.738	2.225	1.732

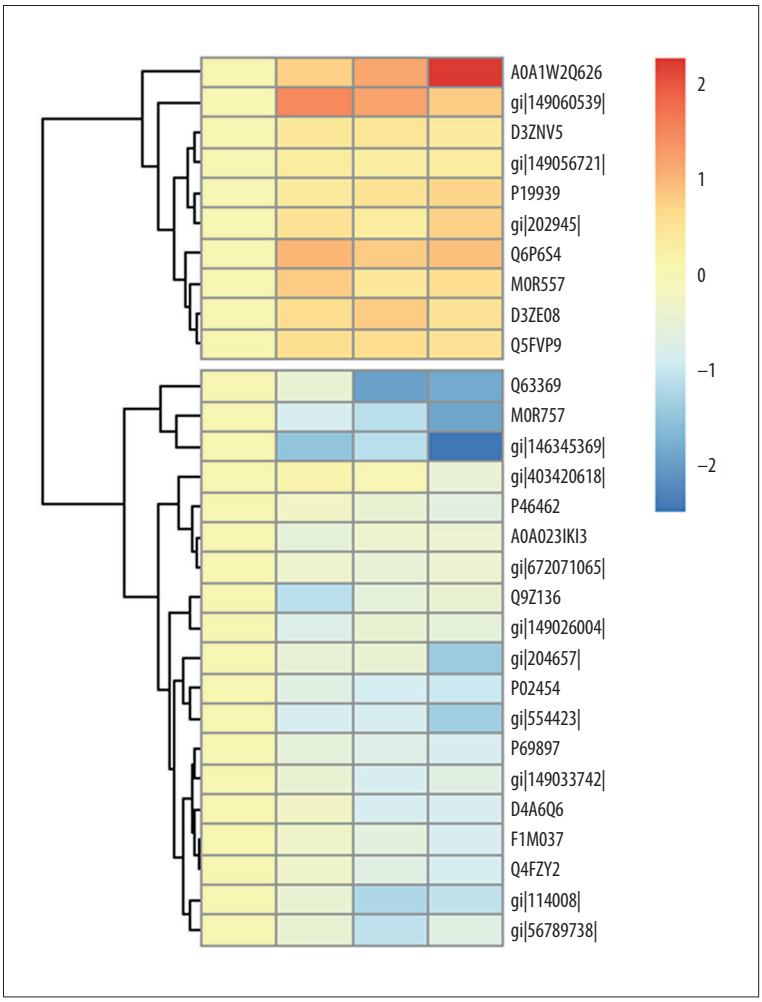

Figure 5. Hierarchical clustering of the differentially-expressed genes. For hierarchical clustering, green and red indicate decreased and increased expression, respectively. Transcripts were clustered by hierarchical clustering using the complete linkage algorithm and Pearson correlation metric in R.

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with the dose of As. Third, As can stimulate aberrant microRNA expression *in vivo* [38], which can interfere with target gene expression, so the level of target proteins may be affected by the As dose. Fourth, since the treatment would stimulate the endogenous response to resist the cytotoxicity caused by As, expression of some target proteins would be associated with the As dose.

We found multiple apolipoproteins associated with As exposure. Several previous studies have described associations between As exposure and apolipoproteins, and it was reported that arsenite exposure, even at the lowest concentration, could significantly increase the plaque size in atherosclerosis. Moreover, As exposure leads to decreased smooth muscle cells and collagen in the plaque. In the apolipoprotein E knock-out model, As exposure was more pro-atherogenic, even at low concentrations, and As made lipid homeostasis more vulnerable to arsenite-induced perturbation in macrophages. This could increase the risk of rupture and myocardial infarction or stroke in humans [39]. In another study, workers exposed to As had Apo-B and Apo-B/Apo-A1 ratios that were significantly higher and the Apo-A1 was significantly lower than in the control subjects, which shows the influence of As on apolipoproteins, suggesting a potential increased risk of As-related diseases [40].

In the present study, we found that the ubiquitin proteasome system (UPS) is another important pathway affected by As exposure. A study by Wang et al. showed that arsenite can bind to the RING finger domain of Rbx1 *in vitro*, which suppresses Cul3-Rbx1 E3 ubiquitin ligase activity, thus hampering Nrf2 ubiquitination and stimulating the Nrf2-involved antioxidant signaling pathway [41]. Hamartin, encoded by TSC1 gene, is an important part of the mTOR pathway, which inhibits the nutrient-mediated or growth factor-stimulated phosphorylation of S6K1 and EIF4EBP1 by negatively regulating mTORC1 signaling [42]. Hamartin was reported to act as a tumor suppressor in melanoma, resulting in hyperactivation of glycogen synthase kinase 3β (GSK3β), followed by phosphorylation of and loss of β-catenin from the nucleus, thereby reducing expression of microphthalmia-associated transcription factor (MITF) and subsequent reductions in tyrosinase and other genes required for melanogenesis [43]. Hamartin appears to be involved in cytotoxicity through the oxidative stress brought by arsenic exposure, as Tsc1-KO animals and cells show features of oxidative stress and impaired antioxidant defenses [44]. Sirtuin6 (SIRT6) has emerged as a critical regulator for modulating angiotensin II-induced injury to the cardiovascular system, and another study demonstrated that SIRT6 overexpression alleviates angiotensin II-induced apoptosis and oxidative stress in vascular endothelial cells by promoting Nrf2 antioxidant signaling [45]. The same mechanism may underlie arsenic toxicity as well. We hope our findings will inspire further research on interactions between arsenite and differentially-expressed proteins.

Conclusions

In summary, by applying 2 proteomic strategies – 2-DE and iTRAQ technology – our results demonstrate that As exposure can alter serum protein profiles in rats compared with controls, which may be detected at the early stage of arseniasis. Further studies are needed to verify the differentially-expressed proteins to provide deeper insights into the early biological effects of As exposure, as well as to define the mechanisms underlying As toxicity.

Conflicts of interest

None.

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