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iTRAQ-based proteomic profiling of human serum reveals down-regulation of platelet basic protein and apolipoprotein B100 in patients with hematotoxicity induced by chronic occupational benzene exposure

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ABSTRACT

Benzene is an important industrial chemical and an environmental contaminant, but the pathogenesis of hematotoxicity induced by chronic occupational benzene exposure (HCOBE) remains to be elucidated. To gain an insight into the molecular mechanisms and developmental biomarkers for HCOBE, isobaric tags for relative and absolute quantitation (iTRAQ) combined with two-dimensional liquid chromatography–tandem mass spectrometry (2D-LC–MS/MS) were utilized. Identification and quantitation of differentially expressed proteins between HCOBE cases and healthy control were thus made. Expressions of selected proteins were confirmed by western blot and further validated by ELISA. A total of 159 unique proteins were identified (≥95% confidence), and relative expression data were obtained for 141 of these in 3 iTRAQ experiments, with fifty proteins found to be in common among 3 iTRAQ experiments. Plasminogen (PLG) was found to be significantly up-regulated, whereas platelet basic protein (PBP) and apolipoprotein B100 (APOB100) were significantly down-regulated in the serum of HCOBE cases. Additionally, the altered proteins were associated with the molecular functions of binding, catalytic activity, enzyme regulator activity and transporter activity, and involved in biological processes of apoptosis, developmental and immune system process, as well as response to stimulus. Furthermore, differential expressions of PLG, PBP and APOB100 were confirmed by western blot, and the clinical relevance of PBP and APOB100 with HCOBE was validated by ELISA. Overall, our results showed that lowered expression of PBP and APOB100 proteins served as potential biomarkers of HCOBE, and may play roles in the benzene-induced immunosuppressive effects and disorders in lipid metabolism.

1. Introduction

Benzene is an important industrial chemical that is also present in gasoline, engine exhausts, wood smoke, and tobacco smoke (IARC, 1989). It is an established cause of acute myeloid leukemia and myelodysplastic syndromes, and may play a role in lymphocytic leukemias and non-Hodgkin lymphoma in humans (Aksoy, 1985; Austin et al., 1988; Hayes et al., 1996, 1997; Savitz and Andrews, 1997; Yin et al., 1996). Moreover, changes in blood and bone marrow consistent with hematotoxicity induced by chronic occupational benzene exposure (HCOBE) are recognized in humans (Lan et al., 2004; Qu et al., 2002; Rothman et al., 1996). Despite extensive research, the exact mechanisms by which benzene exerts its effects remain largely unexplained, and valid biomarkers of HCOBE are needed for clinical diagnosis and drug treatment, as well as the possibility of preventing early adverse effects of benzene exposure.

Emerging technologies such as proteomics have garnered immense interest in terms of their applications, and offer complementary insight into the full complexity of adverse effects of benzene exposure *in vitro* (Lee et al., 2004) and *in vivo* (Joo et al., 2003; Vermeulen et al., 2005). However, results from these researches have not been further validated in large-scale epidemiological investigations. Thus, their identification as direct biomarkers for HCOBE is still far from being generally accepted. Among the proteomic technologies, isobaric tags for relative and

Abbreviations: 2D-LC–MS/MS, two-dimensional liquid chromatography–tandem mass spectrometry; FDR, False Discovery Rate; HCOBE, hematotoxicity induced by chronic occupational benzene exposure; iTRAQ, isobaric tags for relative and absolute quantitation; IPI, International Protein Index; MARS-Hu14, Human 14 Multiple Affinity Removal System; PANTHER, protein analysis through an evolutionary relationships; PSEP, Proteomics System Performance Evaluation Pipeline; PVDF, polyvinylidene fluoride; SCX, strong cation exchange; STEC, short-term exposure concentration.

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absolute quantitation (iTRAQ) reagents (Ross et al., 2004) make it possible to quantitatively screen the entire proteome within the detectable dynamic range for qualitative and quantitative differences in protein expression between individuals and groups differing in exposure history, healthy status and disease. iTRAQ technology has many advantages over other proteomic techniques, such as being high-throughput due to sample multiplexing. In addition, during the tandem mass spectrometric analysis, more than one peptide representing the same protein may be identified, which provides increased confidence in both the identification and quantification of the protein. Furthermore, the technique has been shown to be suitable for the identification of low-abundance proteins such as transcription factors (Aggarwal et al., 2006), which highlight its possible utilization for discovering biomarkers of low-abundant proteins in serum. The advantages of iTRAQ have made it applicable for investigating underlying molecular mechanisms and discovering biomarkers of chemical exposure (Colquhoun et al., 2009; Zhang et al., 2011).

To elucidate the molecular mechanisms of HCOBE and to develop biomarkers that may be of clinical value or early prevention, we utilized iTRAQ proteomic methodology and two-dimensional liquid chromatography–tandem mass spectrometry (2D-LC–MS/MS) to identify and quantitate differentially expressed serum proteins between healthy controls and HCOBE cases. Expressions of selected proteins were confirmed by western blot and further validated using ELISA for their clinical relevance with HCOBE.

2. Materials and methods

2.1. Subjects

The study protocols were conducted according to the principles of the Declaration of Helsinki, and were approved by the Scientific and Medical Ethical Committee of Guangdong Prevention and Treatment Center for Occupational Diseases (GDPTCOD) and Medical Scientific Research Foundation of Guangdong Province, China. All the subjects gave their written informed consent before their inclusion in the study. Patients who met the Chinese National Diagnostic Criteria of Occupational Benzene Poisoning (GBZ 68-2002 and GBZ 68-2008) and were diagnosed with various degrees of occupational benzene poisoning by a panel of occupational physicians in the GDPTCOD were recruited in this study (Table 1). Principles used for diagnosis and the three clinical categories of cases were well described in our previous study (Kuang and Liang, 2005). For iTRAQ proteomic profiles, nine cases were enrolled, including six cases diagnosed with aplastic anemia and three with pancytopenia. The reasons for choosing these cases for iTRAQ analysis were their typical symptoms, clear diagnoses, and the availability of their integrated clinical data. Nine control blood samples were voluntarily donated by healthy, habitual, and controlled blood donors in the community of Guangzhou. Those volunteers were interviewed with free histories of occupational benzene exposure and were matched with cases according to age, gender, and history of smoking and alcoholic drinking. The control subjects were shown to be healthy upon physical examination (Table 1). No patients or controls had occupational histories of exposure to other hematotoxic chemicals or radiation, and were not exposed to radiation therapy or chemotherapy within 15 days before the study. For a validation experiment (ELISA), samples from 30 other cases (7 mild cases, 7 moderate cases and 16 severe cases) and 30 healthy controls (age/sex-matched with cases) were included.

2.2. Sample collection

The patients' blood was drawn on the 2nd day of hospitalization, and the controls' blood on the day of interview. A total 6 ml of blood was separated into two parts: 3 ml blood was allowed to coagulate at room temperature for 30 min and centrifuged at 2000 × *g* for 15 min; the remaining 3 ml blood with heparin anticoagulation was used for blood test. Sera were then aliquoted and stored at −80 °C until use. Hemolysis was not observed during the processes of blood collection. Air samples and benzene-containing solvent samples used at the workplace were also collected by 800B charcoal tubes (Komyo Rikagaku Kogyo K.K, Kanagawa, Japan) and glass containers, respectively, for examining benzene concentration.

2.3. Blood test

The following blood tests were performed in each patient and healthy control: erythrocyte (RBC) count, leukocyte (WBC) count, neutrophil (NEUT) count, platelet (PLT) count, and hemoglobin (HGB), using a MEK-7222K Automatic Hematology Analyzer (Nihon Kohden, Tokyo, Japan).

2.4. Benzene concentration

Benzene short-term exposure concentrations (STEC) (100 ml/min × 15 min) in air at the workplace and benzene concentrations in used solvent materials were analyzed by gas chromatography–mass spectrometry (GC–MS) (6890N gas chromatograph, 5957 Mass Selective Detector, 7683 Automatic Liquid Sampler; Agilent Technologies, Santa Clara, CA, USA).

2.5. Immunodepletion

Stored serum was thawed to room temperature and aliquots of 50 μl were removed for depletion of high-abundance serum proteins using the Human 14 Multiple Affinity Removal System (MARS-Hu14) Spin Cartridges (Agilent Technologies, Wilmington, DE, USA). Samples were diluted to 1:20 with Buffer A (Agilent Technologies) and filtered through a 0.22-μm spin filter (Agilent Technologies) at 2000 × *g* for 1 min. The flow-through fractions were applied onto the spin column which had previously equilibrated with Buffer A, and centrifuged at 100 × *g* for 1 min to elute the depleted proteins. Immunoaffinity columns were incubated for 5 min at room temperature and washed with Buffer A twice, and these fractions were pooled with the initial flow-through. The resulting depleted serum was concentrated using 5000 MWCO cutoff filters (Agilent Technologies). The bound fraction (not used in this study) was then eluted with Buffer B (Agilent Technologies), and the spin cartridge was re-equilibrated with Buffer A for multiple samples. Protein concentration was determined by the Bradford Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as a standard.

2.6. Protein digestion and iTRAQ labeling

Before analysis, a reference pool sample was made of the immunodepleted fractions from all 18 samples (controls and cases) with equal protein amount. Each sample (100 μg) from controls, cases and pool was individually subjected to precipitation using 10% trichloroacetic acid in acetone (−20 °C, overnight) followed by an acetone wash (−20 °C, 15 min). Then the mixture was centrifuged at 12,000 × *g* at 4 °C for 30 min, and the supernatant was removed without disturbing the visible pellet. After being air-dried, the protein pellets were dissolved in the solution buffer, reduced, and blocked with cysteins, as described in the iTRAQ protocol recommended by the manufacturer. The proteins in each individual sample and the pooled sample were digested with sequencing-grade modified trypsin (Sigma, St. Louis, MO, USA) with a protein-to-enzyme ratio of 20:1 at 37 °C overnight, and then labeled separately using the iTRAQ (Applied Biosystems, Foster City, CA, USA) standard protocol for the 8-plex kit. For each group, the samples of controls were labeled with 113, 114 and 116; the samples of cases were labeled with 117, 118, and 119, and the pooled sample was labeled with 121 (Table 2). The labeled digests were then mixed and dried using a rotary vacuum concentrator (Christ RVC 2-25; Osterode am Harz, Germany).

2.7. 2D-LC–MS/MS

The combined peptide mixture was fractionated by strong cation exchange (SCX) chromatography on a 20AD high-performance liquid chromatography (HPLC) system (Shimadzu; Kyoto, Japan) using a polysulfoethyl column (2.1 mm × 100 mm, 5 μm, 200 Å; The Nest Group, Southborough, MA, USA). The mixed sample was diluted with a loading buffer (10 mM KH_2PO_4 in 25% ACN, pH 2.6) and loaded onto the column. Buffer A was identical in composition to the loading buffer, and Buffer B consisted of Buffer A containing 350 mM KCl. Separation was performed using a linear binary gradient of 0–80% Buffer B in Buffer A at a flow rate of 200 μl/min for 60 min. The absorbance at 214 nm and 280 nm was monitored, and a total of 20 SCX fractions were collected along the gradient. These fractions were dried out by the rotary vacuum concentrator, dissolved in Buffer C (5% ACN, 0.1% FA), and analyzed on a QSTAR XL system (Applied Biosystems) interfaced with a 20AD HPLC system (Shimadzu). Peptides were separated on a Zorbax 300SB-C_{18} column (0.1 mm × 150 mm, 5 μm, 300 Å; Microm, Auburn, CA, USA). The HPLC gradient was 5–35% Buffer D (95% ACN, 0.1% FA) in Buffer C at a flow rate of 0.3 μl/min for 70 min. Survey scans were acquired from *m*/*z* 400 to 1800 with up to four precursors selected for MS/MS from *m*/*z* 100 to 2000.

2.8. Analysis of MS/MS data

MS/MS spectra were extracted and searched against the International Protein Index (IPI) database (version 3.45, HUMAN) using ProteinPilot software (version 3.0, revision 114732, Applied Biosystems). Identifications of proteins were only accepted with an unused ProtScore of >1.3 (>95% CI). In addition, accepted protein identifications were required to have a "Local False Discovery Rate (FDR)" estimation of no higher than 5%, as calculated by the PSEP (Proteomics System Performance Evaluation Pipeline) algorithm from the rate of accumulation of "hits" from the Decoy (reversed) database. Following identification, differential expression of proteins between controls and cases was determined by calculating the weighted average