Identification of Urinary Biomarkers Related to Cisplatin-Induced Acute Renal Toxicity Using NMR-Based Metabolomics

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Abstract

Cisplatin is widely used for various types of cancers. However, its side effects, most notably, renal toxicity often limit its clinical utility. Although previous metabolomic studies reported possible toxicity markers, they used small number of animals and statistical approaches that may not perform best in the presence of intra-group variation. Here, we identified urinary biomarkers associated with renal toxicity induced by cisplatin using NMR-based metabolomics combined with Orthogonal Projections to Latent Structures-Discriminant Analysis (OPLS-DA). Male Sprague-Dawley rats (n=22) were treated with cisplatin (10 mg/kg single dose), and the urines obtained before and after treatment were analyzed by NMR. Multivariable analysis of NMR data presented clear separation between non-treated and treated groups. The OPLS-DA statistical results revealed that 1,3-dimethylurate, taurine, glucose, glycine and branched-chain amino acid (isoleucine, leucine and valine) were significantly elevated in the treated group and that phenylacetylglycine and sarcosine levels were decreased in the treated group. To test the robustness of the approach, we built a prediction model for the toxicity and were able to predict all the unknown samples (n=14) correctly. We believe the proposed NMR-based metabolomics with OPLS-DA approach and the resulting urine markers can be used to augment the currently available blood markers.

Key Words: Cisplatin, Toxicity, NMR, OPLS-DA

INTRODUCTION

Cisplatin is the founding member of the platin-group drugs that has platinum metal and ammonium group. Since its first approval in 1978 by US Food and Drug Administration, it has been widely used for various types of cancer such as lung, ovarian, lymphomas, breast and bladder cancers (Smith and Talbot, 1992; von der Maase et al., 2000; Crino et al., 2001; Muggia, 2009). The mechanism of anticancer effect is cytotoxicity due to DNA cross-linking, oxidative damages and apoptosis (Gong et al., 1999; Pruefer et al., 2008). Due to these cytotoxic effect, cisplatin kills not only cancer cells but also normal cells, resulting in undesirable effects in various tissues including kidney, nerves, ear and gastroenteric ones (Loehrer and Einhorn, 1984). Among these, the nephrotoxicity is most common and can be a cause for the cessation of the drug therapy (Arany and Safirstein, 2003; Yao et al., 2007). Typically, Blood Urea Nitrogen (BUN), and blood creatinine are checked to monitor the expression of renal toxicity. In addition, hydration and diuretic measures are taken to minimize possible kidney damages. Still, BUN and creatinine are measured from blood and are late stage kidney functional markers (Hewitt et al., 2004; Davis and Kramer, 2006). Therefore, alternative urine markers may aid clinicians to decide the degree of the toxicity more conveniently.

Evaluation of drug-induced toxicity can be performed in various ways. Direct observation of tissues of interest would provide ultimate answer, but it is often inconvenient or impossible to obtain the tissue without sacrificing the animals. Therefore toxicological markers have been explored that reflect the status of organs. Metabolomics have emerged as a very promising tool for finding and assessing non-invasive markers for drug induced toxicity (Nicholson et al., 1999; Nicholson et al., 2002; Ebbels et al., 2007), because it uses bio-fluids, such as

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blood and urine, to evaluate changes of levels in endogenous small molecules upon drug challenge. It has not only been applied to evaluating drug-induced toxicity, but also to predicting toxicity before drug treatment, opening doors to personalized drug treatment (Clayton et al., 2006; Clayton et al., 2009). With its other application to disease diagnosis and evaluation of prognosis after therapies (Wishart, 2005; Wen et al., 2010b) it is rapidly becoming a valuable tool in many fields of biomedical sciences.

In evaluating kidney toxicities by cisplatin, metabolomics studies were also reported recently (Portilla et al., 2006; Boudonck et al., 2009). One study used NMR combined with Principal Component Analysis (PCA), and another used Mass spectroscopy with Classification and Regression Trees (CART) or logistic regression. Still, these work used less than seven animals for the statistical analysis, and therefore, there is a need to confirm the results with a larger number of animals. Moreover, for the statistical approaches, CART, although it has been used in many metabolomics studies, may not give the best definitive markers for a known property, treated group vs. non-treated group in this case, as it is a non-supervised multivariate analysis. CART or logistic regression may not perform best in the presence of large intra-group variation.

In comparison, Orthogonal Projections to Latent Structures-Discriminant Analysis (OPLS-DA), is a supervised discrimination method that can deal with large intra-group variation, and thus provide markers that can be interpreted straightforwardly (Bylesjo et al., 2006; Wiklund et al., 2008). In addition, the approach gives a prediction model that can be used to test unknown samples for the presence of the property of interest.

Here, we employed NMR-based metabolomics combined with OPLS-DA multivariate analysis to distinguish treated and non-treated group and find associated toxicity markers. Our multivariate model robustly predicted the unknown samples for the presence of toxicity. We believe the proposed NMR-based metabolomics with OPLS-DA approach and the resulting urine markers can be used to augment the currently available blood markers and be applied to assess toxicities associated with other drugs.

**MATERIALS AND METHODS**

**Materials and animals**

Cisplatin was obtained from Sigma Chemical Company (St. Louis, MO). Male Sprague-Dawley rats were obtained from Orient Bio (Sungnam, Korea) at age of 6-7 weeks old. The animals were kept in polypropylene cages with stainless steel grid. Food and water were available freely. The animal rooms were well controlled for balanced humidity and temperature condition with 12 hr light/dark cycle. Animal care and all experimental procedures were conducted in accordance with the guide for animal experiments edited by the Korea Academy of Medical Science, Declaration of Helsinki principle, and approved by the institutional review board. All animal experiments were done at the Inha University Medical school Animal experiment center (Incheon, Korea). Individual animal data are listed in Table S1.

Cisplatin administration and urine sample collection

Rats were randomly divided into dose group and control group. The dose group (n=22) was given 10 mg/kg of cisplatin in saline and the control group (n=8) was treated with just saline by an intraperitoneal injection. Urine samples were collected before (-24 to 0 hrs) and after treatment (72 to 96 hrs) into an ice-cooled jar with a metabolic cage. The collected urine samples were frozen and stored at −80°C until metabolic analysis.

Hematology markers and histological study

After 96 hour treatment, the blood samples were collected using heart puncture before sacrifice. Blood samples were allowed to clot at room temperature and serum was obtained by centrifugation at 13,000 rpm for 10 min for clinical chemistry (BUN and creatinine levels). The kidneys were collected immediately after blood collection, washed with saline buffer and then fixed in 10% formalin. Histological sections of the kidney were stained with hematoxylin and eosin (H&E) stains. Statistical analysis was performed using an unpaired Student’s t-test on SPSS software for Windows (Version 10.0; SPSS, Chicago, IL, USA).

NMR measurement

NMR spectra of the urine samples were measured with an NMR spectrometer (Bruker Biospin, Avance 500) operating at a proton NMR frequency of 500.13 MHz. The acquisition parameters were the same as previously reported (Kang et al., 2008a; Wen et al., 2010b). Two-dimensional NMR spectra were also acquired for identification and structural analysis of the metabolites with a 900 MHz Bruker spectrometer. For proton correlations, Double Quantum Filtered Correlation Spectroscopy (DQF-COSY) data and total correlation spectroscopy (TOCSY) were obtained. For proton and carbon correlation, Heteronuclear Multiple Bond Correlation (HMBC) spectrum and Heteronuclear Single Quantum Coherence (HSQC) spectrum were measured. The experimental parameters are same as previously reported (Kang et al., 2008a; Kang et al., 2008b). We also used Chenomx (Spectral database; Edmonton, Alberta, Canada) for identification of the metabolites. This study used the NMR facility at Korea Basic Science Institute, which is supported by Bio-MR Research Program of the Korean Ministry of Science and Technology (E29070).

Multivariate data analysis

Raw NMR data were Fourier transformed, phase corrected, baseline corrected manually, and normalized against total integration values, 0.025% TSP, and converted to an ascii text file, then binned using in-house written Perl software as previously described (Wen et al., 2010a). For statistical analysis, water and urea regions were excluded. Multivariate statistical analysis was performed using the following softwares: Matlab (MathWorks, Natick, MA), SIMCA-P version 11.0 (Umetrics, Sweden). Orthogonal projections to latent structure-discriminant analysis (OPLS-DA) was performed with one predictive and four orthogonal component (Trygg and Wold, 2002; Bylesjo et al., 2006). 1D projection of Statistical Correlation Spectroscopy (STOCSY) was built by overlaying the color-coded correlation values on to the OPLS-DA variable plot (Cloarec et al., 2005; Maher et al., 2009; Sands et al., 2009).