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ORIGINAL ARTICLE

A proteomic analysis of transplanted liver in a rat model of chronic rejection

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Summary
Background: Chronic rejection (CR) is an important cause of liver allograft failure. In the latter condition, re-transplantation of the liver (ReLT) is the only option for survival. Unfortunately, with the current state of knowledge, it is difficult to diagnose and treat early CR.
Objective: To explore the biomarkers of the chronic rejection in orthotopic liver transplantation (OLT).
Methods: A rat model of chronic liver allograft rejection was established, and the differential protein expression in chronic allograft rejection (CR) was analyzed by iTRAQ-MALDI-TOF/TOF.
Results: Expression of sixty-two proteins was found to be significantly changed in CR rats. In the present study, CLU, Lcn2 and Krt19 were identified and quantified as early and reliable biomarkers for chronic rejection.
Conclusion: Analysis of differential protein expression by iTRAQ-MALDI-TOF/TOF is a potentially effective method to help understand the mechanism of CR in orthotopic liver transplantation. The proteins CLU, Lcn2 and Krt19 might be potential prognostic markers for predicting chronic rejection after liver transplantation.

Introduction

In the past decade, the short-term outcomes and prognoses of organ transplantation have been dramatically improved because of the introduction of the new immunosuppressants, and advances in surgical procedures. However, the long-term prognosis and the rates of possible complications remain unclear [1]. Although the incidence of chronic

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allograft rejection (CR) has decreased from 15—20% to 2—3%, it is still an important cause of liver allograft failure [2—4]. Because CR is a potentially reversible pathologic state, early diagnosis of CR is very important. However, arriving at a histopathologic diagnosis of CR is difficult, it is important to find markers to diagnose and treat CR [5,6].

Proteomic analysis is currently considered to be a powerful tool for the global evaluation of protein expression. Proteomic technology is a systematic overview of protein levels, and has been widely applied to the analysis of proteins in many diseases. Proteomic technology has been used to develop diagnostic, therapeutic, and prognostic biomarkers for some diseases [7—9]. It has also been used to evaluate the reliability, reproducibility, sensitivity, and the dynamic range of protein profiles [10]. Isobaric tag for relative and absolute quantitation (ITRAQ) is a chemical labelling technique based on stable isotopes that allow multiplexing of up to eight different peptide samples [11]. Using this technique, both qualitative and quantitative analyses can be performed. The use of iTRAQ combined with multidimensional liquid chromatography (LC) and tandem mass spectrometer (MS) analysis is emerging as a powerful tool for identification of disease-specific targets and biomarkers [12—16].

Although the proteomic technology has been applied to clinical studies in allograft chronic rejection [17], there have been few reports on liver allograft chronic rejection. In the present study, a rat model of chronic liver allograft rejection was successfully constructed. We applied an iTRAQ-based quantitative proteomic approach (iTRAQ-2DLC-MS/MS) to analyze the protein profiles in rat liver undergoing chronic allograft rejection in order to obtain a deeper understanding of the underlying mechanisms of chronic rejection, and to identify reliable biomarkers of chronic rejection.

Materials and methods

Animals

Male Sprague-Dawley (SD) rats were purchased from the Laboratory Animal Center in Fujian Medical University. Male Lewis rats were purchased from Shanghai Slac Laboratory Animal Co. Ltd, specific pathogen free, license: SCXK2007-0005. All donor and recipient rats were about 8 to 10 weeks old and weighed 200—250 g. The rats were housed in conventional cages at 20—22 °C with free access to water and food with a 12 h light—dark cycle. Animals were examined twice daily.

All procedures involving laboratory animal use were performed in accordance with the guidelines and approved by the animal care and use committee of Fujian Medical University.

Liver transplantation and pathological examination

A syngenic control group (group I) consisted of SD-to-SD OLT ($n = 10$). An allogeneic group (group II) consisted of SD-to-Lewis OLT ($n = 10$). We have previously established an animal model of chronic rejection after liver transplantation, in which the livers from SD transplanted to Lewis rats treated with immunosuppressants, exhibited chronic rejection 120 days after operation [18]. In the present study, all the rats were treated as previously described, and eight rats in each group were sacrificed 120 days after operation. The specimens from the right lobe of the transplanted livers were prepared for pathological examination (evaluated by two independent, blinded pathologists). The chronic rejection activity index was determined according to the early- and late-stage pathological criteria of chronic rejection proposed by the 5th Banff Conference [19]. Partial tissues of the grafts were collected and placed in freezing vials, rinsed by the saline, and then stored at −80 °C.

Sample preparation

All tissue samples were thawed on ice. Samples taken from the same liver lobe of different rats were pooled in equal amounts. Samples were cut into small pieces and washed with PBS. The tissues were homogenized with lysis buffer containing 7 M urea, 2 M thiourea, 0.1% PMSF and 0.5% DTT in a glass homogenizer on ice [20]. The resulting homogenate was suspended and centrifuged for 30 min at 12,000 rpm at 4 °C. The supernatants were collected and protein concentrations were measured by the Bradford assay using bovine serum albumin (BSA) as standard [21]. The supernatants were stored at −80 °C for further study.

Samples (100 μg) were digested with trypsin, and labelled individually with one iTRAQ tag (Applied Biosystems, USA) as follows: rats without CR, iTRAQ 114 (IT114), and with CR iTRAQ 116 (IT116). The labelled samples were then pooled and dried by centrifugal evaporation.

Off-line 2D LC-MS/MS

Chromatographic separation of the pooled samples was performed using a 20AD high-performance liquid chromatography (HPLC) system (Shimadzu; Kyoto, Japan). The combined peptide mixture was fractionated by strong cation exchange liquid chromatography (SCX) using a 0.5 × 23 mm, 5 μm, 300 Å Column (Waters, USA). Samples were added to loading buffer (10 mM KH_2PO_4 in 25% acetonitrile, pH 2.6) and loaded into the column. Buffer A was the same as the loading buffer, and the component of buffer B was same with that of the 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 [22]. The absorbance at 214 nm and 280 nm was monitored, and a total of 25 SCX fractions were collected along the gradient.

The fractions were dried by a rotary vacuum concentrator, dissolved in buffer C (5% acetonitrile, 0.1% FA) and analyzed on a QSTAR XL system (Applied Biosystems) interfaced with a 20AD HPLC system (Shimadzu). Peptides were separated on a reverse phase (RP) column, Zorbax 300SB-C18 column (Agilent Technologies). 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 90 min [22]. Survey scans were acquired from m/z 400—1800 with up to four precursors selected for MS/MS from m/z 100—2000. Each SCX fraction was analyzed in duplicate.