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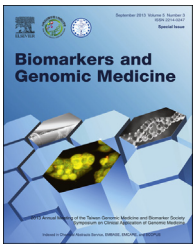

SHORT COMMUNICATION

Utilized mass spectrometry-based protein profiling system to identify potential biomarkers of hepatocellular carcinoma

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Abstract Hepatocellular carcinoma (HCC) is the most common malignant liver tumor. The purpose of this study is to characterize proteins secreted from the HepG2 cells, which may relate to cell differentiation and tumor metastasis. In the proteomic analysis, the secretome was identified by nano-high–performance liquid chromatography/electrospray ionization tandem mass spectrometry (nano-HPLC/ESIMS/MS) followed by peptide fragmentation pattern analysis. In this study, three proteins, p130Cas-associated protein (p130Cas/BCAR1), TAR DNA-binding protein 43 (TDP43/TARDBP) and translationally controlled tumor protein (TCTP/TPT1), were identified and confirmed by Western blotting, which showed significantly

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differential expression compared with the normal liver cells. Analyzing differential protein expressions in HepG2 cell by proteomic approaches suggests that p130Cas/BCAR1, TDP43/TARDBP and TCTP/TPT1 as key proteins and may serve as biomarkers for HCC.

Introduction

Hepatocellular carcinoma (HCC) is the most or second most common cause of cancer-related mortality, especially in Asia and Africa, with a 5-year survival rate of less than 5% without treatment.[1] A chronic infection with the hepatitis B virus or hepatitis C virus increases the risk of developing HCC.[2,3] Measurement of serum alpha-fetoprotein (AFP) is widely used as a serology tumor marker for HCC, with a level exceeding 500 mg/L considered to be a positive indicator.[4] Unfortunately, not all HCCs (e.g., the fibrolamellar type) secrete AFP, and levels of it are often elevated during pregnancy, in tumors of gonadal origin, and even during acute or chronic viral hepatitis (chronic hepatitis B or chronic hepatitis C).[5,6] Biomarker identification remains a major challenge in current HCC research for early diagnosis.[7]

Secreted proteins, referred to as the secretome, are an important class of proteins that control and regulate a number of biological and physiological processes, making it a clinically relevant source for biomarkers and therapeutic target discoveries. To enhance our understanding of the hepatocellular carcinoma proteome, secretome of HepG2 cells was analyzed by proteomic approaches. This study is designed to establish an optimal technique for a proteomic map of hepatocellular carcinoma proteins. The database provides not only information obtained from HepG2 cell secretome proteins of hepatocellular carcinoma, but also potential diagnostic protein biomarkers for further investigation.

Materials and methods

Cell culture

HepG2 (liver tumor cell) and CCL-13 (liver normal cell) cells were maintained at 37 °C and 5% CO_2 in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT, USA), 1% penicillin/streptomycin (Gibco) and 44mM $NaHCO_3$. After 3 days, the cells were washed with phosphate buffered saline (PBS) and the medium was replaced with serum-free RPMI 1640 medium for 12 hours.

Sample preparation

After incubation with serum-free RPMI 1640 medium, the secreted proteins in the medium of HepG2 and CCL-13 cells were centrifuged at 1500 *g* for 10 minutes at 4 °C. The supernatants were filtered by 0.8-μm filter and the protein concentrations were adjusted to 1 mg/mL by 25mM ammonium bicarbonate.

Cell secretome samples (100 μL) were reduced, alkylated, and then digested with trypsin using standard protocols. Two microliters of formic acid were added to each sample prior to the mass spectrometric analysis for protein identification.

Proteomic analysis

The complex peptide mixtures were separated by reverse-phase high—performance liquid chromatography/electro spray ionization tandem mass spectrometry (RP-nano-HPLC-ESIMS/MS). The protein tryptic digests were fractionated using a flow rate of 400 nL/min with a nano-HPLC system

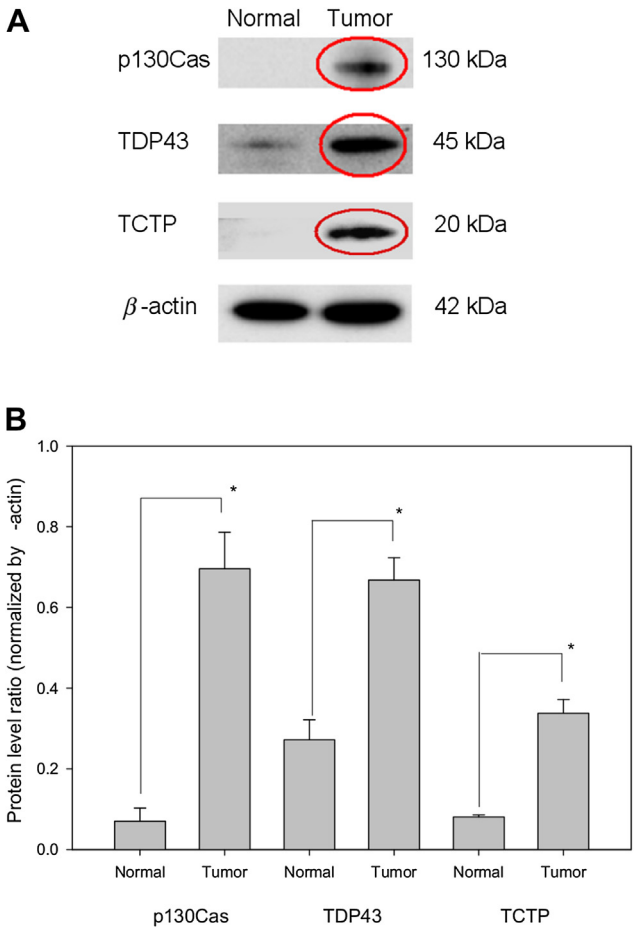

Figure 1 (A) Immunoreactive bands of p130Cas/BCAR1, TDP43/TARDBP, TCTP/TPT1 and β-actin from HepG2 and CCL-13 cells. (B) Protein expression data are calculated after normalizing with β-actin. Data are expressed as ratio of mean ± standard error and the asterisk denotes a significant difference compared with CCL-13 at $p < 0.05$ ($N = 4$).

(nanoACQUITY UPLC, Waters, Milford, MA, USA) coupled to an ion trap mass spectrometer (LTQ Orbitrap Discovery Hybrid FTMS, Thermo, San Jose, CA, USA) equipped with an electrospray ionization source. For RP-nano-HPLC-ESI-MS/MS, a sample (2 μL) of the desired peptide digest was loaded into the reverse phase column (Symmetry C18, Waters, Milford, MA, USA, 5 μm, 180 μm × 20 mm) by autosampler. The RP separation was performed using a linear acetonitrile gradient from 99% buffer A (100% D.I. water/0.1% formic acid) to 85% buffer B (100% acetonitrile/0.1% formic acid) in 100 minutes using the micropump. The separation is performed on a C18 microcapillary column (BEH C18, Waters, Milford, MA, USA, 1.7 μm, 75 μm × 100 mm) using the nano separation system. As peptides eluted from the microcapillary column, they were electrosprayed into the ESI MS/MS with the application of a distal 2.1 kV spraying voltage with heated capillary temperature of 200 °C. Each cycle of one full scan mass spectrum (m/z 400—2000) was followed by four data dependent tandem mass spectra with collision energy set at 35%.

Database search

For protein identification, Mascot software (version 2.2.1; Matrix Science, London, UK) was used to search the Swiss-Prot human protein sequence database. Positive protein identifications were defined when Mowse scores greater than 100 were considered significant ($p < 0.05$). Proteins were annotated by similar searches using UniProtKB/Swiss-Prot databases.

Western blot

Western blot analysis was applied to detect candidate proteins, and quantitative analysis of Western blotting was carried out using the ImageQuant-TL-7.0 software, version 2010 (Amersham Biosciences, Belgium). Proteins were separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, with 100 μg of protein loaded in each lane. Gels were equilibrated for 10 minutes in a transfer buffer and then electroblotted on nitrocellulose membranes for 75 minutes at 100 V. Membranes were incubated with 1 μg/mL of primary antibodies for 60 minutes and with a 1:10,000 dilution of secondary antibody for 30 minutes. Next, the membranes were exposed to an X-ray film prior to later development. Afterward, the membranes were stripped and probed with anti-β-actin antibodies for normalization. The protein—protein interaction pathways were performed by String 9.0 Web software.

Results

In this study, we performed proteomic approaches to identify differently secreted proteins between HepG2 cell and CCL-13 cell. A database search initially suggested 213 differentially expressed proteins. Most of them were identified at minimal confidence level in which only one unique peptide sequence matched, whereas 21 protein identifications

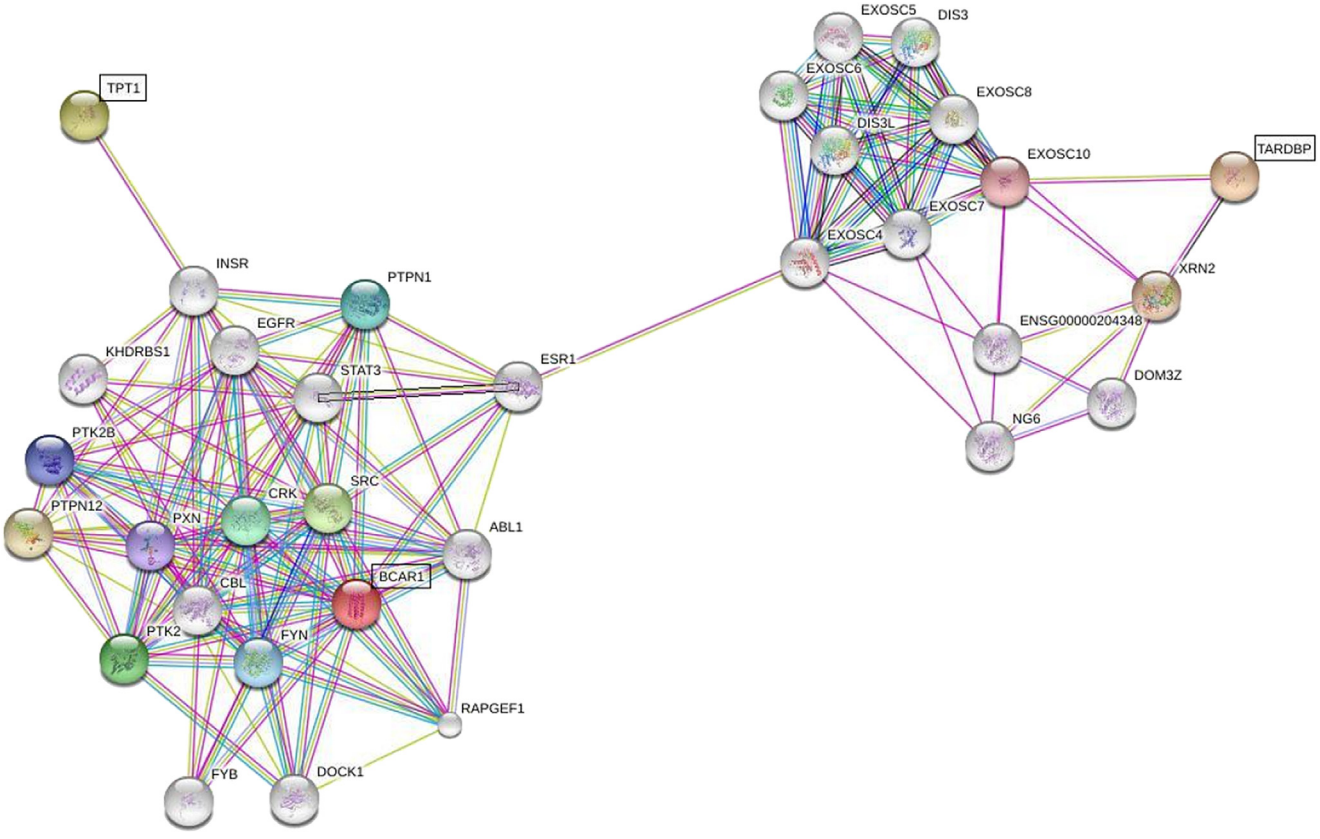

Figure 2 The protein-protein interaction networks were analyzed using String 9.0 Web software. Three proteins, p130Cas/BCAR1, TDP43/TARDBP, and TCTP/TPT1, are marked by boxes.

showed higher confidence levels with at least three unique peptide sequences matched. Three of 21 proteins exhibited significantly higher levels expressed in the culture medium of HepG2 cells compared with that of CCL-13 cells. The three proteins including p130Cas/BCAR1, TDP43/TARDBP, and TCTP/TPT1 were involved in cell differentiation and cell cycle regulation. The results of Western blotting showed that expression of p130Cas/BCAR1, TDP43/TARDBP, and TCTP/TPT1 in HepG2 cells was at least 1.5-folds ($p < 0.05$) of that in CCL-13 cells (Fig. 1). The protein-interacted networks analyzed by String 9.0 Web software showed the interacted proteins of p130Cas/BCAR1, TDP43/TARDBP, and TCTP/TPT1 in cells (Fig. 2). Thus, the biological significance between increase in the secretion of the three proteins and liver tumor progression could be further studied accordingly.

Discussion

The protein constitutively secreted by a particular type of cells *in vitro* is referred to as the secretome. In this study, we use the mass spectrometry technique as an analysis method for determining liver cancer biomarkers on tumor cell secretome. The cell model was used to identify the tumor progression-related proteins, which are secreted into the serum-free culture medium. Secreted proteins may coordinate many cellular processes in tumor cells, including growth, division, differentiation, apoptosis, migration, and adhesion, thereby contributing to the growth and spread of the tumor cells. Thus, the identification of secreted proteomics in tumor cells provides potential biomarkers closely related to carcinogenesis. Moreover, disease-specific protein biomarkers allow us to define prognosis of disease and gain deep insight into disease mechanisms by which proteins play a major role. Studies on secretomes from different tumor cells may find novel biomarkers. In this study, our data suggest that p130Cas/BCAR1, TDP43/TARDBP, and TCTP/TPT1 notably express and secrete into the culture medium of HepG2 cells. The p130Cas was identified by Matsuda[8] in 1990 and originally introduced as a highly phosphorylated protein in cell transformations of the Src (pp60v-Src) and Crk (p47gag-Crk) oncogenes. It was a key signal molecule of the integrin pathway that was included in cell proliferation, apoptosis, and migration mediated by integrin.[9] TDP43, a messenger RNA (mRNA)-binding protein, played an important role in the regulating mRNA metabolism, which was involved in transcriptional repression, exon skipping, and RNA splicing.[10] TCTP was initially identified in mammalian tumor cells and controlled the posttranscriptional modification. It was multi-involved in division cellular processes, such as apoptosis, microtubule organization, ion homeostasis, and interacting proteins in the PLK1 protein kinase.[11] Thus, the potential utility of the three proteins as liver cancer biomarkers merits study in the future.

Acknowledgments

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