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Comparative proteomics of cancer stem cells in osteosarcoma using ultra-high-performance liquid chromatography and Orbitrap Fusion mass spectrometer

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ABSTRACT

Osteosarcoma is the most common malignant tumour found in bones, and it has a poor prognosis. For improved therapy, it is significant to have a deep understanding of the proteomics changes in the cancer stem cells (CSCs) of osteosarcoma. Therefore, a comparative proteomics approach based on ultra-high-performance liquid chromatography coupled to an Orbitrap Fusion mass spectrometer (UHPLC-Orbitrap Fusion MS) was established to investigate the key molecular changes between CSCs and non-CSCs in human osteosarcoma HOS cells. A proteomic analysis was performed on these samples and a total of more than 6600 proteins were identified in each run. Moreover, most of the correlation coefficients between three biological repeats were more than 0.9 in each group. That finding demonstrated not only that the reproducibility of the test is good but also that the stability of this MS is excellent. A label-free quantitative method was applied to analyse differentially expressed proteins. Using the criteria of greater than 1.5-fold changes and a p value < 0.05, 124 proteins were identified as being significantly different between HOS-CSCs and non-CSCs. A pathway analysis of differentially expressed proteins by Ingenuity Pathway Analysis (IPA) revealed the potential molecular regulatory networks that may regulate CSCs. Selected differential α-actinin 4 (ACTN4) proteins were validated by Western blot assay. These findings enhance our understanding of the molecular changes in CSCs and may provide additional improvements in therapy for treating osteosarcoma. Moreover, the UHPLC-Orbitrap Fusion MS-based proteomics method is helpful in cancer research.

1. Introduction

Osteosarcoma is the most common malignant tumour found in bones, and it has a poor prognosis [1,2]. In patients with metastasis, the 5-year survival rate is no more than 30% [3]. Thus, this illness has become a heavy health burden in our society. More and more studies have shown that, as in many tumours, cancer stem cells (CSCs) are the root of development, recurrence and metastasis in osteosarcoma [4]. Additionally, CSCs usually have a CD133-positive phenotype and can be isolated by flow sorting [4,5]. Because of the relatively small amount of CSCs and the difficulty of separating them, little is known about their biological characteristics, especially the molecular mechanisms underlying their pathogenesis. That limit would hinder the development of new therapeutic methods. Because proteins play direct roles in biological processes, it is vital to screen key proteins associated with malignant features of CSCs in osteosarcoma.

Proteomics, an emerging field of -omics science that combines advanced analytical technology with new methods of calculation, can accurately identify and quantify thousands of proteins in multiple biological samples [6,7]. There are two primary analytical techniques used in proteomics, namely protein chip-based proteomics and mass spectrometry (MS)-based proteomics. Of course, both strengths and weakness are presented by each method. Protein chip-based proteomics, which are also called microarray-based proteomics, capture molecules (such as proteins, antigens, antibodies, etc.) on the surface of solid carriers to detect protein molecules that interact with specific molecules [8,9]. This approach is characterized by being fast and having high throughput, high automation and good miniaturization [10,11]. However, there may be a phenomenon of non-characteristic combinations that results in false-positive detection [12]. Thus, MS-based proteomics technology, including label-based and label-free approaches, has been the mainstream technology for large-scale protein

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identification and quantitation [13,14]. There are many label-based proteomics approaches, such as using isotope-coded affinity tags (ICAT) [15], isobaric tags for relative and absolute quantification (iTRAQ) [16], tandem mass tags (TMT) [17] and stable isotope labelling with amino acids in cell culture (SILAC) [18]. Although they can result in the highly accurate quantification of multiple samples, these methods are relatively expensive and time-consuming. At present, the label-free methods are becoming more and more popular in proteomics because there is no real limit to their sample size, and they have easy operation and reliable quantification performance [19].

Prior to the MS detection of proteins, sample preparation is another vital issue associated with the depth and extent of proteome analysis [20]. Historically, the most common technique for protein separation has been electrophoresis consisting of two-dimensional gel electrophoresis (2-DE) and capillary electrophoresis [21]. This method primarily depends on the molecular weight and charge of proteins, and it has several inevitable limitations including relatively larger sample sizes, a time consuming process and difficulty in distinguishing proteins with low abundance or small molecular weights [21,22]. Currently, separation techniques using various liquid chromatography (LC) variations are popular because they can be high throughput, accurate and provide the fast separation of complex samples. In particular, ultra-high-performance liquid chromatography (UHPLC), which can stand up to high pressures, may greatly enhance the proteome coverage within a shorter time [23].

In this study, an UHPLC-Orbitrap Fusion MS-based comparative proteomics approach was established to explore the key molecular changes between CSCs and non-CSCs in human osteosarcoma HOS cells. After a statistical analysis, differentially expressed proteins in HOS-CSCs were identified. An annotation of the cellular components and biological pathways was further explored. Furthermore, the biological events were validated by conventional molecular biology. The results not only demonstrate the advantage of UHPLC- Orbitrap Fusion MS-based proteomics strategy, but it also helped improve the therapy for osteosarcoma.

2. Experimental

2.1. Reagents and materials

High-performance liquid chromatography (HPLC)-grade formic acid, acetonitrile, dithiothreitol and iodoacetamide were commercially obtained from the Sigma-Aldrich Company (Sigma-Aldrich, China). A MilliQ system (Millipore, USA) was used to obtain distilled water. Hank's balanced saline solution (HBSS), trypsin, and Dulbecco's Modified Eagle's Medium (DMEM) culture medium were purchased from Corning Corporation (Grand Island, NY). Foetal bovine serum (FBS), streptomycin sulphate and penicillin were purchased from Gibco Corporation (Grand Island, NY). Phycoerythrin (PE)-conjugated anti-human CD133 antibody was purchased from Miltenyi Corporation (Miltenyi Biotech Inc., Germany). Human osteosarcoma cell line HOS was obtained from the Type Culture Collection at the Chinese Academy of Sciences, Shanghai.

2.2. Cell culture and fluorescence-activated cell sorting (FACS)

Human osteosarcoma cells were cultured in DMEM supplemented with 10% FBS, penicillin (100 U/mL) and a streptomycin (100 μg/mL) cocktail. The cells were kept under standard adherent conditions at 37 °C in a 5% CO_2-humidified air atmosphere. HOS-CSCs and non-CSC fractions were isolated by FACS. In brief, the cells were washed with HBSS and detached from the plates using trypsin. After the samples were centrifuged at 4 °C, the cell pellets were re-suspended in HBSS supplemented with 1% FBS. The cells were then stained with anti-human CD133 antibody and incubated for 10 min in a 4 °C incubator. After the staining, the cells were re-suspended in HBSS containing 1% FBS and sorted by a FACS Aria III (BD Biosciences, USA).

2.3. Sample preparation

The proteins from the sorted cells were extracted with 8 M urea, and 100 μg of protein was reduced by adding 5 mM dithiothreitol for 0.5 h at 56 °C and alkylated by adding 20 mM iodoacetamide for 0.5 h at room temperature in the dark. After that, 5 mM dithiothreitol was added and the samples were kept in the dark for 15 min. The protein samples were finally transferred to a 10 kDa filter unit, washed with 8 M urea and 50 mM ammonium bicarbonate twice, digested with trypsin at a mass ratio of 1:50 enzyme/protein overnight at 37 °C and then the digestion was stopped by adding 1% formic acid.

2.4. LC-MS/MS conditions

Dried peptide samples were re-dissolved in solvent A (0.1% formic acid in water). A liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed with Orbitrap Fusion MS (Thermo Fisher Scientific) equipped with an online Easy-nLC 1000 system (Thermo Fisher Scientific). The injected peptides were separated on a reverse-phase nano-HPLC C18 column (Pre-column: 3 μm, 120 Å, 2 cm × 100 μm ID; analytical column: 1.9 μm, 120 Å, 10 cm × 100 μm ID) at a flow rate of 400 nL/min with a 75-min gradient of 5–30% solvent B (0.1% formic acid in acetonitrile), and a series of adjusted linear gradients was applied according to the hydrophobicity of the eluted fractions. For peptide ionization, 2000 V was applied and a 320 °C capillary temperature was used. For the Oribitrap Fusion MS detection, a precursor scan was performed in the Orbitrap by scanning from m/z 300–1400 with a resolution of 120,000 (at m/z 200), a targeted automatic gain control (AGC) value of 5e5 and a maximum injection time of 50 ms. The ions selected under top-speed mode were isolated in Quadrupole with an isolation width of 1.6 Da, and they were fragmented by higher energy collision-induced dissociation (HCD) with a normalized collision energy of 32%, then measured in the linear ion trap. The typical MS/MS scan conditions were as follows: a targeted AGC value of 5e3, a maximum fill time of 35 ms and the dynamic exclusion was employed for 18 s.

2.5. Data analysis

The acquired MS/MS spectra were searched using MaxQuant (version 1.5.6.5) against the National Center for Biotechnology Information (NCBI) human RefSeq protein databases (updated on 04-07-2013) as previously described [24]. The parameter settings were as follows: the mass tolerances were 10 ppm for precursors and 0.5 Da for product ions; two missed cleavages were allowed; the dynamic modifications were acetyl (protein N-term) and oxidation (M); a false discovery rate (FDR) of 1% was applied at the peptide and protein levels; the enzyme specificity was set to “Trypsin”; a minimum number of seven amino acids were required for peptide identification; default settings were used for variable and fixed modifications (variable modification, acetylation (N terminus) and methionine oxidation; fixed modification, carbamidomethylation); and the proteins and protein isoforms that could not be distinguished by unique peptides were grouped into protein groups.

2.6. Data mining and bioinformatics analysis

To explore the differentially expressed proteins between the two cell subtypes, t-tests were used. A p value below 0.05 was regarded as a significant difference. The canonical signalling pathways of differentially expressed proteins were analysed using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, http://www.ingenuity.com/). Gene ontology (GO) analysis for cellular components and biological networks was performed with STRING version 10.5 (http://string-db.org/) [25].

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