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Proteomic profiling of isogenic primary and metastatic medulloblastoma cell lines reveals differential expression of key metastatic factors

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ABSTRACT

Medulloblastoma is the most common malignant brain tumor in children. Around 30% of medulloblastoma patients are diagnosed with metastasis, which often results in a poor prognosis. Unfortunately, molecular mechanisms of medulloblastoma metastasis remain largely unknown. In this study, we employed the recently developed deep proteome analysis approach to quantitatively profile the expression of >10,000 proteins from CHLA-01-MED and CHLA-01R-MED isogenic cell lines derived from the primary and metastatic tumor of the same patient diagnosed with a group IV medulloblastoma. Using statistical analysis, we identified ~1400 significantly altered proteins between the primary and metastatic cell lines including known factors such as placental growth factor (PLGF), LIM homeobox 1 (LHX1) and prominim 1 (PROM1), as well as the negative regulator secreted protein acidic and cysteine rich (SPARC). Additional transwell experiments and immunohistochemical analysis of clinical medulloblastoma samples implicated yes-associated protein 1 (YAP1) as a potential key factor contributing to metastasis. Taken together, our data broadly defines the metastasis-relevant regulated proteome and provides a precious resource for further investigating potential mechanisms of medulloblastoma metastasis.
Significance: This study represented the first deep proteome analysis of metastatic medulloblastomas and provided a valuable candidate list of altered proteins in metastatic medulloblastomas. The primary data suggested YAP1 as a potential driver for the metastasis of medulloblastoma. These results open up numerous avenues for further investigating the underlying mechanisms of medulloblastoma metastasis and improving the prognosis of medulloblastoma patients.

1. Introduction

Medulloblastoma is the most common central nervous system (CNS) tumor in children and the cause of many pediatric oncology-related deaths [1]. With the standard protocol, comprising surgery, craniospinal radiotherapy and chemotherapy, overall survival rates of patients diagnosed with medulloblastoma have reached 70–80% [2,3]. However, a number of patients survive with serious complications, such as a decline in cognition and intellect, endocrine dysfunction and an increased incidence of secondary neoplasms, which have not markedly improved over the past decades [4,5]. The prognosis of these patients is largely dependent on the risk-adapted scheme at diagnosis including age, size of the residual disease and/or status of metastasis. Presence of metastasis often results in a less favorable outcome for patients with medulloblastomas and unfortunately, approximately 25–33% of patients are diagnosed with metastasis [6,7]. This has become a major barrier to further improve the prognosis of patients with medulloblastomas.

The molecular mechanisms that drive medulloblastoma metastasis remain elusive. Expression changes of some proteins have been shown to contribute to medulloblastoma metastasis, such as α9β1 integrin, SPARC and PDGFRa [6]. Several molecular pathways have been shown to promote medulloblastoma metastasis, including extracellular matrix formation, the intracellular network of filaments and growth factor signaling [8–11]. For the latter, overexpression of ERBB2, IGF-R as well as c-MET has been shown to associate with invasion of medulloblastomas [12]. A recent study revealed that placental growth factor (PLGF) and its receptor neuropilin 1 (Nrp1) were overexpressed in the majority of medulloblastomas, independent of their subtype, which could promote the spread of medulloblastomas [8]. Despite these significant advances, most attempts to treat metastatic medulloblastomas by targeting only one or two proteins have not been successful [4,5]. A global understanding of molecular mechanisms underlying the metastatic behavior of medulloblastomas would be a further step towards the design of more effective therapies.

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Genome-wide profiling of gene expression alterations has provided global insight into the genetic regulation of medulloblastoma metastasis [13]. Proteomics, however, could provide more direct evidence of metastasis-relevant proteins and identify therapeutic targets for developing novel therapeutic strategies [14,15]. Recent development of novel strategies and advances in mass spectrometry has enabled deep proteome analysis (>8000 gene products) of any given human cell line or tissue within a reasonable time [16]. Unfortunately, this high-throughput technique has not been exploited to study metastatic events in medulloblastomas thus far. To this end, we adapted a deep proteome approach to quantitatively analyzing a primary CHLA-01-MED and metastatic CHLA-01R-MED isogenic cell lines derived from a patient with a Group IV medulloblastoma [17]. Quantitative profiling and statistical analysis of >10,000 proteins revealed 551 upregulated and 886 downregulated proteins in metastatic cells, representing the first quantitative deep proteome analysis of a metastatic medulloblastoma. These included medulloblastoma metastasis-associated proteins such as PLGF, ERBB2 and SPARC, as well as numerous novel candidates. Bioinformatic analysis further revealed several signaling pathways involved in cancer metastasis, which led us to investigate the role of YAP1, the key factor of the Hippo pathway in medulloblastomas. Transwell experiments revealed that the metastatic ability of CHLA-01R-MED cells was significantly reduced after knocking down YAP1 using siRNA. Immunohistochemical analysis further confirmed the differential expression of YAP1 in clinical medulloblastoma samples. Together, our data demonstrates the potential role of YAP1 in the medulloblastoma metastasis process.

2. Material and methods

2.1. Cell line and cell culture

CHLA-01-MED and CHLA-01R-MED cells were purchased from ATCC and cultured accordingly (http://www.atcc.org/). Briefly, cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Life Technologies Corp., Carlsbad, CA) supplemented with1x B27 (Life Technologies Corp., Carlsbad, CA), 20 ng/ml basic FGF (Life Technologies Corp., Carlsbad, CA) and 20 ng/ml EGF(Life Technologies Corp., Carlsbad, CA) at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

2.2. Protein extraction and in-solution digestion and peptides preseparation

Cells were washed with the pre-cold phosphate-buffered saline (PBS, Mediatech Inc., Manassas, VA) and collected in chilled lysis buffer, 8 M urea in 100 mM NH_4HCO_3 with 1 × protease inhibitor cocktail (Roche Diagnostic GmbH, Germany) and incubated on ice for half an hour. Cell debris were removed by centrifugation and the supernatant was collected.Protein concentration in lysates was determined by the Bradford assay accordingly. The extracted proteins were digested by trypsin as previously described [18]. Briefly, after the alkylation and reduction, protein lysate was diluted four times with 100 mM NH_4HCO_3 (pH 8.0) to decrease the urea concentration to 2 M. Trypsin was then added at an enzyme-substrate ratio of 1:100 (w/w). The digestion was subsequently performed at 37 °C for 16 h.

2.3. HPLC/MS/MS analysis

Tryptic digest was then pre-separated into 20 fractions with a reverse phase C18 Xbridge column (Waters Corp., Milford, MA) by a preparative HPLC system (Shimadzu, Japan) at high pH condition. Each fraction was lyophilized and further separated using a manually packed reversed phase C18 column (170 mm × 79 μm, 3-μm particle size, Dikma, China) coupled to Easy nLC 1000 (Thermo Fisher Scientific, Waltham, MA). The separated peptides were ionized using NSI source, then analyzed in an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, Waltham, MA) with a top speed 3 s data-dependent mode. For full MS spectra, the intact peptides with m/z 350–1300 were detected in the Orbitrap at a resolution of 120,000 at m/z 200 with automatic gain control (AGC) 5×10^5. For MS/MS scan, ions with intensity above 5000 and charge state 2–6 in each full MS spectrum were sequentially fragmented by Higher Collision Dissociation (HCD) with normalized collision energy of 32%. The dynamic exclusion duration was set to be 60 s, and the precursor ions were isolated by quadrupole with isolation window 1 Da. The fragment ions were analyzed in ion trap with AGC 7000 at rapid scan mode. The raw spectra data was processed by Maxquant software (v1.4.1.2) [19]. MS/MS spectra data was searched against the Uniprot human database (88,817 sequences) by Andromeda search engine [20]. Contaminants and reversed versions of all sequences were enabled with the following search parameters: carbamidomethylation of cysteine residues as fixed modification and acetyl (Protein N-term), oxidation (M) as variable modifications. Trypsin was specified as the proteolytic enzyme with maximum 2 missing cleavages. Seven ppm was used for main search of the precursor ions while fragment ion mass tolerance was set to 0.6 Da. The maximum false discovery rate (FDR) for proteins and peptides was 0.01 and a minimum peptide length of six amino acids was required.

2.4. Bioinformatic Analysis

Perseus (v1.5.6. 0) was further employed for the further data processing [21]. The contaminate proteins and reverse sequence hits were excluded and two sample *t*-test analysis and Benjamini-Hochberg false discovery rate (FDR) for multiple test correction was used to identify the significantly expressed proteins between these two cell lines. The biological process (BP), molecular function (MF), cellular component (CC) and KEGG pathways analysis of the significantly expressed proteins was performed using Database for Annotation, Visualization and Integrated Discovery Bioinformatics Database (DAVID 6.7) [22].

2.4.1. Antibodies and immunoblot analysis

Anti-MMP2 (Cat.13132), Actin (cat. 12,620), YAP1(cat.12395) antibodies were purchased from Cell signaling technology, anti-CDC5L(cat. Sc-81,220), anti-Filamin-A (cat. Ab76289) and anti-PLGF(cat. Ab140639) antibodies were from Santa Cruz and Abcom, respectively. For immunoblot analysis, 30 micrograms of lysate was separated with SDS-PAGE gels and then transferred to PVDF membranes. The membranes were blocked for 1 h in blocking solution at room temperature and probed overnight at 4 °C with the primary antibodies. After washing with PBST (PBS with 0.1% Tween-20), the membranes were incubated with secondary antibody coupled with horseradish peroxidase for 1 h. Immunoreactivity was visualized using enhanced chemiluminescence reagents (Life Technologies Corp., Carlsbad, CA).

2.4.2. siRNAs and cell transfection

The sequence of the siRNA against YAP1 was as purchased from Genomeditech (Shanghai, China) and the sequence as follows: hs-YAP1-si-1: GGUCCUCUUCCUGAUGGAU; hs-YAP1-si-2: CCGUUUCCCAGACUACCUU; hs-YAP1-si-3: CUGCCACCAAGCUAGAUAA. Cell transfection was performed as before, briefly, cell were suspended with 400 μL IMDM medium containing ×B27 (Life Technologies Corp., Carlsbad, CA), 20 ng/ml basic FGF (Life Technologies Corp., Carlsbad, CA) and 20 ng/ml EGF (700 μL). 50 μL OPTI-DMEM (Life Technologies Corp., Carlsbad, CA) with 100 nM siRNA and 50 μL OPTI-DMEM with 1 μL Lipo2000 (Life Technologies Corp., Carlsbad, CA) were mixed and added to the cells. After 6hs, 200 μL of the cells were added to the upper chamber and 700 μL of the culture medium was added to the lower chamber. After growing for 72 h, the cells in the lower chamber were counted.

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