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Top-down proteomic characterization of DAOY medulloblastoma tumor cell line

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ABSTRACT

The proteome of the DAOY medulloblastoma cell line has been investigated by an LC–MS top-down platform. This approach, unlike bottom-up ones, allows identifying proteins and peptides in their intact/native forms, disclosing post-translational modifications, proteoforms and naturally occurring peptides. Indeed, 25 out of the 53 proteins identified, were not previously characterized in DAOY cells. Most of them were functionally interconnected, being mainly involved in binding, catalytic and structural activities, and metabolic processes. The top-down approach, applied in this preliminary study, disclosed the presence of several naturally occurring peptide fragments that characterize DAOY cells.

1. Introduction

Medulloblastoma represents the most frequent malignant brain tumor in pediatric patients, whereas it is generally less frequent in adult. Medulloblastoma is typically located in the posterior cranial fossa and has a neuroectodermal origin [1]. The molecular mechanisms underlying the etiopathogenesis of the disease are largely unknown. Different studies pursued the proteomic profiling of medulloblastoma through *in vitro* characterization of the available cell lines, namely DAOY, D283, ONS76 and UW228 [2–8].

In particular, Peryl et al. [2] analyzed DAOY and D283 cell pellets by two-dimensional gel electrophoresis (2-DE) coupled with MALDI-time-of-flight-MS (MALDI-TOF-MS). This approach yielded 332 proteins and particularly relevant was the detection in both cell lines of the antiapoptotic Ded protein possibly responsible for medulloblastoma aggressive behaviour [2].

In another study, proteomic profiling was used to study the modifications of protein expression in DAOY cells overexpressing the neurotrophin receptor TrkC [3]. TrkC signaling is involved in regulation of cell differentiation, proliferation and survival, and TrkC negative tumors are generally associated with a poor prognosis. Upon induction with the TrkC ligand neurotrophin-3, transfected cells overexpressed 13 proteins involved in tumor proliferation, migration and invasiveness, compared to control untransfected cells. These data possibly suggested potential targets to be exploited to improve the response to therapy and to reduce tumor aggressiveness [3]. The same authors described the characterization in DAOY cells of two proteoforms of the superoxide dismutase (Mn-SOD or SOD2) enzyme, sharing identical sequence, but different oxidative states [4]. The tandem MS analysis of the relative 2-DE digested spots by MALDI-TOF/TOF, Qq-TOF and ion trap, with both CID and ETD fragmentations, identified the presence of oxidation at Trp-186 in both forms and additionally at His-30 and His-31 in only one of them, possibly responsible for a decreased SOD-2 activity [4].

Recently, Cappellari et al. [5] applied a multi-disciplinary and quantitative approach to study the expression of ecto-5′NT/CD73 ectonucleotidase, an enzyme involved in tumor malignancy,

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in primary (DAOY, ONS76) and metastatic (D283) medulloblastoma cell lines. The enzyme was expressed in DAOY and ONS76 cell lines but only weakly in the D283, suggesting its involvement in tumor aggressiveness and poor prognosis. 2-DE/MALDI-TOF-MS was also applied to study the proteome of DAOY, UW228 and ONS76 medullospheres [6]. Compared with cells grown in adherence, the DAOY and UW228 medullospheres expressed different proteomic profiles with immature stem cell features. Conversely, spheres derived from ONS76T cells, a most immature medulloblastoma cell line, showed only quantitative variations in protein expression levels, compared to corresponding adherent cells. It is noteworthy to point out the identification, in all medullospheres, of nucleophosmin, which could plausibly be correlated with the capacity of medulloblastoma to survive in adverse tissue environments [6].

Recently, the proteomic characterization of exosomes released by D283, DAOY and UW228 cells allowed the identification of numerous protein species involved in cell migration and modulation of the immune response, suggesting a possible role of extracellular vesicles in tumor aggressiveness and progression [7]. Furthermore, SDS-PAGE and MALDI-TOF analysis of exosomes from DAOY, UW228 and ONS76 grown in adherence and as medullosphere, allowed the characterization of 33 proteins, including several iron carrier proteins, evidencing a possible role of iron in tumor aggressiveness and proliferation [8]. More recently, a proteomic approach was exploited to study the proteome of the Human Sonic Hedgehog (SHH) medulloblastoma subgroup (exhibiting constitutive aberrant activation of the SHH pathway) before and after retinoic acid-induced differentiation [9]. The results of this study indicated that 68 proteins were differently modulated in induced-versus-control cells, the heat shock protein 70 being overexpressed in non-induced cells [9].

Overall, to date, all studies reporting the proteomic characterization of medulloblastoma cell lines have been based on bottom-up approaches, thus identifying proteins after enzymatic digestion.

The present study provides the first attempt to investigate the medulloblastoma DAOY cell proteome in its intact protein state, using a top-down LC–MS proteomic strategy. This approach is particularly suitable for the identification of small proteins (<25 kDa) and peptides. In addition, through the analysis of naturally occurring forms, it allows identifying PTMs, fragment peptides, isoforms and proteoforms, that may represent key molecules associated to the onset and progression of human disorders. The present data could provide important contributions to the definition of the medulloblastoma molecular signature, plausibly including potential therapeutic targets and diagnostic biomarkers.

2. Material and methods

2.1. Reagents and chemicals

All organic solvents employed in this study were of LC–MS grade purity. Acetonitrile (ACN), methanol, water, and formic acid (FA) were purchased from Merck (Darmstadt, Germany). 2,2,2-trifluoroacetic acid (TFA) was purchased from Mallinckrodt Baker B.V. (Deventer, The Netherlands). Ultrapure water for sample pretreatment was obtained through the P.Nix Power System apparatus, (Human, Seoul, Korea). The protease-inhibitor cocktail (AEBSF, Aprotinin, Bestatin, E-64, EDTA, and Leupeptin) was purchased from Sigma-Aldrich (Buchs, Switzerland). Media and supplements for cell culture were purchased from Euroclone (Milan,Italy).

2.2. Instrumentation

HPLC-ESI–MS/MS top-down analyses were performed on an UltiMate RSLCnano System (ThermoFisher Scientific, San Jose, CA, USA) coupled to Orbitrap Elite high resolution mass spectrometry detector (ThermoFisher) with ESI source. Reverse-phase chromatography was performed on 150 × 1.0 mm Zorbax 300 SB-C8 3.5 μm (Agilent Technologies, Santa Clara, CA, USA) columns.

2.3. Sample pretreatment

200,000 DAOY cells were plated in 6-well plates, using complete standard growth medium (high glucose Dulbecco's Modified Eagle's Medium, 1% Pen/Strep, 1% L-Glutamine, 10% Fetal Bovine Serum). 24 h after plating the cell monolayer was washed twice in PBS, and starved in serum-free DMEM for 24 h. Thereafter, the cells were detached by use of trypsin and collected for proteomic analysis. To this aim, mechanical disruption of the cell membranes was performed after acidification in 0.1% TFA (v/v) solution mixed with the protease-inhibitor cocktail: cell pellets underwent three cycles of freezing, thawing and sonication. The resulting solution was then lyophilized, suspended in 50 μL of 0.1% (v/v) TFA aqueous solution, and subsequently analyzed by HPLC-ESI-Orbitrap Elite-MS.

2.4. HPLC-ESI-Orbitrap Elite-MS analysis

Top-down HPLC–MS/MS chromatographic analyses were performed using an aqueous solution of FA (0.1%, v/v) as eluent A and ACN/water mixture (80:20, v/v) 0.1% FA (v/v) as eluent B. The following step gradient was applied: (i) from 0 to 5% of eluent B (2 min), (ii) from 5 to 70% of eluent B (38 min), (iii) from 70% to 99% of eluent B (5 min), (iv) 99% eluent B (6 min), at a flow rate of 50 μL/min, followed by column reconditioning, from 99 to 5% B (2 min) to 5% eluent B (10 min). In order to prevent ESI source contamination from sample salts, MS acquisition in the positive ion mode started 4 min after sample injection. MS spectra were collected using *Full Scan* acquisition mode, in the 350–2000 m/z range, with the resolving power set to 60000. MS/MS analyses were performed in Data-Dependent Scan (DDS) mode, by selecting and fragmenting the five most intense multiply-charged ions of the *Full Scan* spectra by Collision Induced Dissociation (CID, 35% normalized collision energy), with a resolving power of 60000. The minimum signal intensity required was 500.0; the isolation width was 5 m/z, default charge state +2, activation Q 0.25 and activation time 10 ms. The DDS set parameters were as follows: repeat count 1, repeat duration 30 s, exclusion list size 500, exclusion duration 15 s, exclusion mass width relative to reference mass: low 10 ppm, high 10 ppm, minimum signal threshold (counts) 500, default charge state 2, isolation width 5 (m/z), activation Q 0.250, activation time 10 msec.

The tuning parameters of the ESI source were as follows: capillary temperature 300 °C, source voltage 4.0 kV, sheath gas 12, auxiliary gas 0, S lens RR level 60%. The injected volume was 20 μL.

2.5. MS and MS/MS spectra data analysis

High resolution MS and MS/MS data were elaborated both manually, using Xcalibur (version 2.0.7 SP1, Thermo Fisher Scientific) deconvolution software, and automatically through the Proteome Discoverer 1.4.0 software (2013, Thermo Fisher Scientific). The analysis was performed using the SEQUEST HT cluster search engine against the Swiss Prot Homo Sapiens proteome (Uniprot_homo + sapiens_reviewed, released on August 2014).

The setting parameters were as follows: minimum precursor mass 300 Da; maximum precursor mass 10000 Da; total intensity threshold 0.0; minimum peak count 5; Signal to Noise (S/N) threshold 3; precursor mass tolerance 10 ppm; fragment mass tolerance 0.6 Da, use average precursor mass "false", use average