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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

Table 1
Proteins and peptides identified in medulloblastoma DAOY cells by top-down LC-ESI-Orbitrap Elite MS/MS proteomic analysis.

Accession[a,b]	Description[a]	Identification in medulloblastoma cell lines	Σ Coverage	# Peptides	MW [kDa]
P07737	**Profilin-1**	–	89.29	6	15.0
	Identified peptides	**MH⁺ [Da]**	**RT**		
	GTCQDAAIVGYKDSPSVWAAVPGKTFVNITPAEVGVLVGKDRSSF	4637.395	29.74		
	RTKSTGGAPTFNVTVTKTDKTLVLLMGKEGVHGGLINKKCYEMASHLRRSQY	5722.011	25.31		
	RTKSTGGAPTFNVTVTKTDKTL	2323.260	19.96		
	MGKEGVHGGLINKKCYEMASHLRRSQY	3092.525	20.48		
	GTCQDAAIVGYKDSPSVW	1896.880	24.48		
	YVNGLTLGGQKCSVIRDSLLQDGEFSMD	3045.466	28.10		
P62937	**Peptidyl-prolyl *cis*-trans isomerase A**	[6]	24.85	8	18.0
	Identified peptides	**MH⁺ [Da]**	**RT**		
	DIAVDGEPLGRVSFE	1603.793	25.22		
	FDIAVDGEPLGRVSFE	1750.863	27.53		
	ILKHTGPGILSMANAGPNTNGSQF	2425.227	23.93		
	IAVDGEPLGRVSFE	1488.768	24.57		
	FDIAVDGEPLGRVSFEL	1863.949	30.70		
	VDGEPLGRVSFE	1304.649	22.63		
	DIAVDGEPLGRVSFEL	1716.876	28.76		
	AVDGEPLGRVSFE	1375.685	22.92		
P60709	**Actin, cytoplasmic 1**	[7][d], [8], [8][d]	5.33	2	41.7
	Identified peptides	**MH⁺ [Da]**	**RT**		
	WISKQEYDESGPSIVHRKCF	2409.166	21.25		
	ISKQEYDESGPSIVHRKCF	2223.089	19.31		
P60174	**Triosephosphate isomerase**	[6]	15.38	2	30.8
	Identified peptides	**MH⁺ [Da]**	**RT**		
	IGEKLDEREAGITEKVVFEQTKVIADNVKDWSKVVL	4086.217	31.71		
	VDIINAKQ	900.514	16.84		
P09382	**Galectin-1**	[6][c]	19.26	2	14.7
	Identified peptides	**MH⁺ [Da]**	**RT**		
	LTVKLPDGYEFKFPNRLNL	2264.243	30.74		
	ITFDQANLTVKLPDGYEFKFPNRLNL	3053.624	31.12		
P68104	**Elongation factor 1-alpha 1**	–	15.80	4	50.1
	Identified peptides	**MH⁺ [Da]**	**RT**		
	GNVAGDSKNDPPMEAAGFTAQ	2076.929	20.63		
	VIILNHPGQISAGYAPVLDCHTAHIACK	2941.517	24.43		
	FVPISGWNGDNMLEPSANMPWFKG	2694.244	31.32		
	GNVAGDSKNDPPMEAAGF	1776.785	21.76		
P02545	**Prelamin-A/C**	[6][c], [3]	5.57	2	74.1
	Identified peptides	**MH⁺ [Da]**	**RT**		
	TCGQPADKASASGSGAQVGGPISSGSSASSVTVTR	3165.505	18.59		
	CGTCGQPADKASASGSGAQVGGPISSGSSASSVTVTR	3325.530	18.91		
P23528	**Cofilin-1**	[2] [3]	16.87	1	18.5
	Identified peptides	**MH⁺ [Da]**	**RT**		
	VKMLPDKDCRYALYDATYETKESKKEDL	3352.641	22.07		
P23527	**Histone H2B type 1-O**	–	22.22	1	13.9
	Identified peptides	**MH⁺ [Da]**	**RT**		
	VRLLLPGELAKHAVSEGTKAVTKYTSSK	2983.694	24.95		
P62805	**Histone H4**	[8][d]	39.81	1	11.4
	Identified peptides	**MH⁺ [Da]**	**RT**		
	LENVIRDAVTYTEHAKRKTVTAMDVVYALKRQGRTLYGFGG	4627.456	28.39		
Q9BQE3	**Tubulin alpha-1C chain**	[2]	6.46	2	49.9
	Identified peptides	**MH⁺ [Da]**	**RT**		
	YCLEHGIQPDGQMPSDKTIGGGDDSFNTF	3129.355	25.10		
	EHGIQPDGQMPSDKTIGGGDDSFNTF	2750.198	23.52		
Q8NC51	**Plasminogen activator inhibitor 1 RNA-binding protein**	–	4.66	2	44.9
	Identified peptides	**MH⁺ [Da]**	**RT**		
	PGHLQEGFGCVVTNRF	1760.851	24.59		
	PGHLQEGFGCVVTNRFDQL	2117.015	26.42		

Table 1 (*Continued*)

Accession[a,b]	Description[a]	Identification in medulloblastoma cell lines	Σ Coverage	# Peptides	MW [kDa]
P10809	**60 kDa heat shock protein, mitochondrial**	[2], [7][d], [8]	5.41	2	61.0
	Identified peptides	**MH⁺ [Da]**	**RT**		
	VVVTEIPKEEKDPGMGAMGGMGGGMGGGMF	2926.325	26.43		
	EVVVTEIPKEEKDPGMGAMGGMGGGMGGGMF	3055.365	26.77		
P07910	**Heterogeneous nuclear ribonucleoproteins C1/C2**	[2]	6.54	2	33.6
	Identified peptides	**MH⁺ [Da]**	**RT**		
	YYDRMYSYPARVPPPPPIAR	2409.208	22.54		
	YSYPARVPPPPPIAR	1680.921	21.05		
Q99497	**Protein DJ-1**	[6], [3]	12.17	1	19.9
	Identified peptides	**MH⁺ [Da]**	**RT**		
	AIVEALNGKEVAAQVKAPLVLKD	2376.386	24.54		
P27797	**Calreticulin**	[6]	2.88	1	48.1
	Identified peptides	**MH⁺ [Da]**	**RT**		
	WQVKSGTIFDNF	1441.711	27.75		
P62158	**Calmodulin**	–	9.40	2	16.8
	Identified peptides	**MH⁺ [Da]**	**RT**		
	MIREADIDGDGQVN	1532.700	18.61		
	IREADIDGDGQVN	1401.662	17.12		
P52272	**Heterogeneous nuclear ribonucleoprotein M**	[2],[7][d]	3.56	2	77.5
	Identified peptides	**MH⁺ [Da]**	**RT**		
	QGGGGGGGSVPGIER	1284.629	16.44		
	VPAGMGAGLER	1057.546	18.42		
Q9H1E3	**Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1**	–	12.35	1	27.3
	Identified peptides	**MH⁺ [Da]**	**RT**		
	KVVDYSQFQESDDADEDYGRDSGPPTKKIR	3445.618	19.23		
P06733	**Alpha-enolase**	[2], [6], [7][d]	8.99	1	47.1
	Identified peptides	**MH⁺ [Da]**	**RT**		
	LKVNQIGSVTESLQACKLAQANGWGVMVSHRSGETEDTF	4191.069	27.95		
P05787	**Keratin, type II cytoskeletal 8**	[7][d]	4.97	2	53.7
	Identified peptides	**MH⁺ [Da]**	**RT**		
	AVVVKKIETRDGKLVSESSDVLPK	2597.487	19.69		
	DGKLVSESSDVLPK	1473.781	19.42		
P24534	**Elongation factor 1-beta**	–	7.11	1	24.7
	Identified peptides	**MH⁺ [Da]**	**RT**		
	GFGDLKSPAGLQVLND	1630.844	25.38		
Q01105	**Protein SET**	[7][d]	10.00	1	33.5
	Identified peptides	**MH⁺ [Da]**	**RT**		
	FVNHPQVSALLGEEDEEALHYLTRVEVTE	3324.642	29.91		
P05783	**Keratin, type I cytoskeletal 18**	–	7.21	1	48.0
	Identified peptides	**MH⁺ [Da]**	**RT**		
	SLGSVQAPSYGARPVSSAASVYAGAGGSGSR	2854.400	21.50		
P63241	**Eukaryotic translation initiation factor 5A-1**	–	6.49	1	16.8
	Identified peptides	**MH⁺ [Da]**	**RT**		
	AAVAIKAMAK	973.586	16.42		
Q15942	**Zyxin**	–	2.62	1	61.2
	Identified peptides	**MH⁺ [Da]**	**RT**		
	FTCHQCAQQLQGQQF	1766.776	21.75		
Q96AE4	**Far upstream element-binding protein 1**	[2]	3.11	1	67.5
	Identified peptides	**MH⁺ [Da]**	**RT**		
	YYAQTSPQGMPQHPPAPQGQ	2182.995	18.81		
Q32P51	**Heterogeneous nuclear ribonucleoprotein A1-like 2**		5.63	1	34.2
	Identified peptides	**MH⁺ [Da]**	**RT**		

Table 1 (*Continued*)

Accession[a,b]	Description[a]	Identification in medulloblastoma cell lines	Σ Coverage	# Peptides	MW [kDa]
	NNQSSNFGPMKGGNFGGR	1868.846	19.13		
P0C6T2	**Dolichyl-diphosphooligosaccharide–protein glycosyltransferase subunit 4**	–	100.00	1	4.2
	Identified peptides	**MH⁺ [Da]**	**RT**		
	MITDVQLAIFANMLGVSLFLLVVLYHYVAVNNPKKQE	4191.281	48.30		
P63313	**Thymosin beta-10**	–	97.73	3	5.0
	Identified peptides	**MH⁺ [Da]**	**RT**		
	A(Acetyl)DKPDMGEIASFDKAKLKKTETQEKNTLPTKETIEQEKRSEIS	4934.537	20.90		
	A(Acetyl)DKPDMGEIASFDKAKLKKTETQEKNTLPTKETIEQEKRSE	4734.412	20.49		
	LKKTETQEKNTLPTKETIEQEKRSEIS	3188.704	17.17		
P62328	**Thymosin beta-4**	–	97.73	4	5.0
	Identified peptides	**MH⁺ [Da]**	**RT**		
	S(Acetyl)DKPDMAEIEKFDKSKLKKTETQEKNPLPSKETIEQEKQAGES	4961.501	19.82		
	S(Acetyl)DKPDMAEIEKFDKSKLKKTETQEKNPLPSKETIEQEKQAG	4745.421	19.89		
	LKKTETQEKNPLPSKETIEQEKQAGES	3070.588	16.77		
	TETQEKNPLPSKETIEQEKQAGES	2701.320	17.56		
P31949	**Protein S100-A11**	–	99.05	4	11.7
	Identified peptides	**MH⁺ [Da]**	**RT**		
	A(Acetyl)KISSPTETERCIESLIAVFQKYAGKDGYNYTLSKTEFLSFMNTELAAFTKNQ KDPGVLDRMMKKLDTNSDGQLDFSEFLNLIGGLAMACHDSFLKAVPSQKRT	11644.768	39.15		
	IAVFQKYAGKDGYNYTLSKTEF	2543.280	24.27		
	IGGLAMACHDSF	1221.537	24.27		
	IGGLAMACHDSFLKAVPSQKRT	2330.211	23.37		
P40926	**Malate dehydrogenase, mitochondrial**	[2]	13.91	3	35.5
	Identified peptides	**MH⁺ [Da]**	**RT**		
	AKVAVLGASGGIGQPLSL	1637.958	26.18		
	AKVAVLGASGGIGQPLSLLLKNSPLVSRLTL	3072.844	33.89		
	SLVDAMNGKEGVVECS	1637.750	22.24		
P30041	**Peroxiredoxin-6**	[2]	29.02	2	25.0
	Identified peptides	**MH⁺ [Da]**	**RT**		
	PGGLLLGDVAPNFEANTTVGRIRFHDFLGDSWGIL	3754.940	34.03		
	PGGLLLGDVAPNFEANTTVGRIRFHDFLGDS	3285.675	30.01		
P60468	**Protein transport protein Sec61 subunit beta**	–	25.00	1	10.0
	Identified peptides	**MH⁺ [Da]**	**RT**		
	PGPTPSGTNVGSSGRSPSKAVAAR	2238.157	15.56		
P63279	**SUMO-conjugating enzyme UBC9**	–	24.68	1	18.0
	Identified peptides	**MH⁺ [Da]**	**RT**		
	LNEPNIQDPAQAEAYTIYCQNRVEYEKRVRAQAKKFAPS	4539.288	24.68		
P62263	**40S ribosomal protein S14**	–	21.85	1	16.3
	Identified peptides	**MH⁺ [Da]**	**RT**		
	APRKGKEKKEEQVISLGPQVAEGENVFGVCHIF	3623.902	28.23		
P08670	**Vimentin**	[2], [6], [3]	13.30	16	53.6
	Identified peptides	**MH⁺ [Da]**	**RT**		
	DGQVINETSQHHDDLE	1836.799	17.08		
	IKTVETRDGQVINETSQHHDDLE	2664.284	17.76		
	LIKTVETRDGQVINETSQHHDDLE	2777.369	18.89		
	MFGGPGTASRPSS	1251.579	17.39		
	NLRETNLDSLPLVDTHSKR	2208.170	21.82		
	NLRETNLDSLPLVDTHSKRTLLIK	2776.567	23.88		
	NLRETNLDSLPLVDTHSKRTLLIKTVETRDGQVINETSQHHDDLE	5180.648	23.97		
	RETNLDSLPLVDTHSKR	1981.045	20.19		
	RETNLDSLPLVDTHSKRTLLIK	2549.439	22.98		
	RETNLDSLPLVDTHSKRTLLIKTVETR	3135.735	23.09		
	RETNLDSLPLVDTHSKRTLLIKTVETRDGQVINETSQHHDDLE	4953.527	23.49		
	RRMFGGPGTASRPSSSR	1806.913	14.46		
	TLLIKTVETR	1173.721	19.85		
	TLLIKTVETRDGQVINETSQHHDDLE	2991.500	22.31		
	TVETRDGQVINETSQHHDD	2180.981	15.67		
	TVETRDGQVINETSQHHDDLE	2423.109	17.64		
P04406	**Glyceraldehyde-3-phosphate dehydrogenase**	[5], [6][c], [7][d], [8]	19.40	6	36.0

Table 1 (*Continued*)

Accession[a,b]	Description[a]	Identification in medulloblastoma cell lines	Σ Coverage	# Peptides	MW [kDa]
	Identified peptides	**MH⁺ [Da]**	**RT**		
	FQYDSTHGKFHGTVKAENGKLVINGNPITIFQERDPSKIKWGDAGAEY	5363.670	25.49		
	ISWYDNEFGYSNRVVD	1963.882	25.69		
	ISWYDNEFGYSNRVVDL	2076.966	28.30		
	QERDPSKIKWGDAGAEY	1949.935	19.94		
	YDNEFGYSNRVVD	1577.690	20.67		
	YDNEFGYSNRVVDL	1690.768	24.70		
P84243	**Histone H3.3**	–	40.53	2	15.3
	Identified peptides	**MH⁺ [Da]**	**RT**		
	RFQSAAIGALQEASEAYL	1924.978	28.17		
	FEDTNLCAIHAKRVTIMPKDIQLARRIRGERA	1109.574	23.10		
P67809	**Nuclease-sensitive element-binding protein 1**	[6][c]	10.80	1	35.9
	Identified peptides	**MH⁺ [Da]**	**RT**		
	RRPENPKPQDGKETKAADPPAENSSAPEAEQGGAE	3658.746	15.04		
P06748	**Nucleophosmin**	[6][c]	9.18	1	32.6
	Identified peptides	**MH⁺ [Da]**	**RT**		
	SIRDTPAKNAQKSNQNGKDSKPSSTPR	2912.505	6.13		
Q13263	**Transcription intermediary factor 1-beta**	[2]	1.32	1	88.5
	Identified peptides	**MH⁺ [Da]**	**RT**		
	SGEGEVSGLMR	1121.526	19.12		
P43243	**Matrin-3**	[2]	5.43	2	94.6
	Identified peptides	**MH⁺ [Da]**	**RT**		
	DSQGHGRDLSAAGIGLLAAATQSLSMPASLGR	3108.579	30.85		
	GNLGAGNGNLQGPR	1324.672	16.61		
O43390	**Heterogeneous nuclear ribonucleoprotein R**	[2]	1.58	1	70.9
	Identified peptides	**MH⁺ [Da]**	**RT**		
	YQDTYGQQWK	1316.596	16.71		
P22626	**Heterogeneous nuclear ribonucleoproteins A2/B1**	[2]	17.85	5	37.4
	Identified peptides	**MH⁺ [Da]**	**RT**		
	GGPYGGGNYGPGGSGGSGGYGGR	1944.825	17.57		
	NMGGPYGGGNYGPGGSGGSGGYGGR	2189.906	19.01		
	GGGNYGPGGSGGSGGYGGR	1570.664	15.17		
	GGGPGGGNFGGSPGYGGGR	1564.687	17.52		
	NQQPSNYGPMKSGNFGGSR	2025.920	18.09		
P04908	**Histone H2A type 1-B/E**	–	82.31	2	14.1
	Identified peptides	**MH⁺ [Da]**	**RT**		
	VLLPKKTESHHKAKGK	1801.083	4.75		
	ILELAGNAARDNKKTRIIPRHLQLAIRNDEELNKLLGKVTIAQGGVLPNIQA	5696.263	27.39		
P06454	**Thymosin alpha-11**	–	100.00	1	3.8
	Identified peptides	**MH⁺ [Da]**	**RT**		
	S(Acetyl)DAAVDTSSEITTKDLKEKKEVVEEAENGRDAPAN	3788.834	21.32		
S4R457	**Heterogeneous nuclear ribonucleoprotein K**	[5], [9]	48.05	1	8.8
	Identified peptides	**MH⁺ [Da]**	**RT**		
	ETEQPEETFPNTETNGEFGKRPAEDMEEEQAFKRSE	4087.810	23.10		
P06454-2	**Prothymosin alpha isoform 2**	–	99.09	1	12.0
	Identified peptides	**MH⁺ [Da]**	**RT**		
	S(Acetyl)DAAVDTSSEITTKDLKEKKEVVEEAENGRDAPANGNANEENGEQEADNEVDEEEEEGGEEEEEEEEGD GEEEDGDEDEEAESATGKRAAEDDEDDDVDTKKQKTDEDD	11978.902	21.64		
Q15056	**Eukaryotic translation initiation factor 4H**	–	13.30	1	27.4
	Identified peptides	**MH⁺ [Da]**	**RT**		
	TVATPLNQVANPNSAIFGGARPREEVVQKEQE	3449.776	23.82		
P0CG48 P0CG47	**Ubiquitin (1–76)**	–	97.23	1	8.5
	Identified peptides	**MH⁺ [Da]**	**RT**		
	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG	8560.638	30.42		

Table 1 (*Continued*)

Accession[a,b]	Description[a]	Identification in medulloblastoma cell lines	Σ Coverage	# Peptides	MW [kDa]
Q9UNM1	**Chaperonin 10-related protein**	–	75.26	1	10.3
	Identified peptides	**MH⁺ [Da]**	**RT**		
	VERSAAETVTKGGIMLPEKSQGKVLQATVVAVGSGSKGKGGEIQPVSVKVGDKVLLPEYGGTKWLDDKDYFL	7557.081	26.46		a

[a] Bold letters (accession n. and sequences) refer to proteins and peptide fragments identified in DAOY cells culture in serum-free medium after 24, 48 and 72 h.
[b] Underlined accessions refer to proteins not previously identified in DAOY cells.
[c] Medullospheres.
[d] Medulloblastoma cell exosomes.

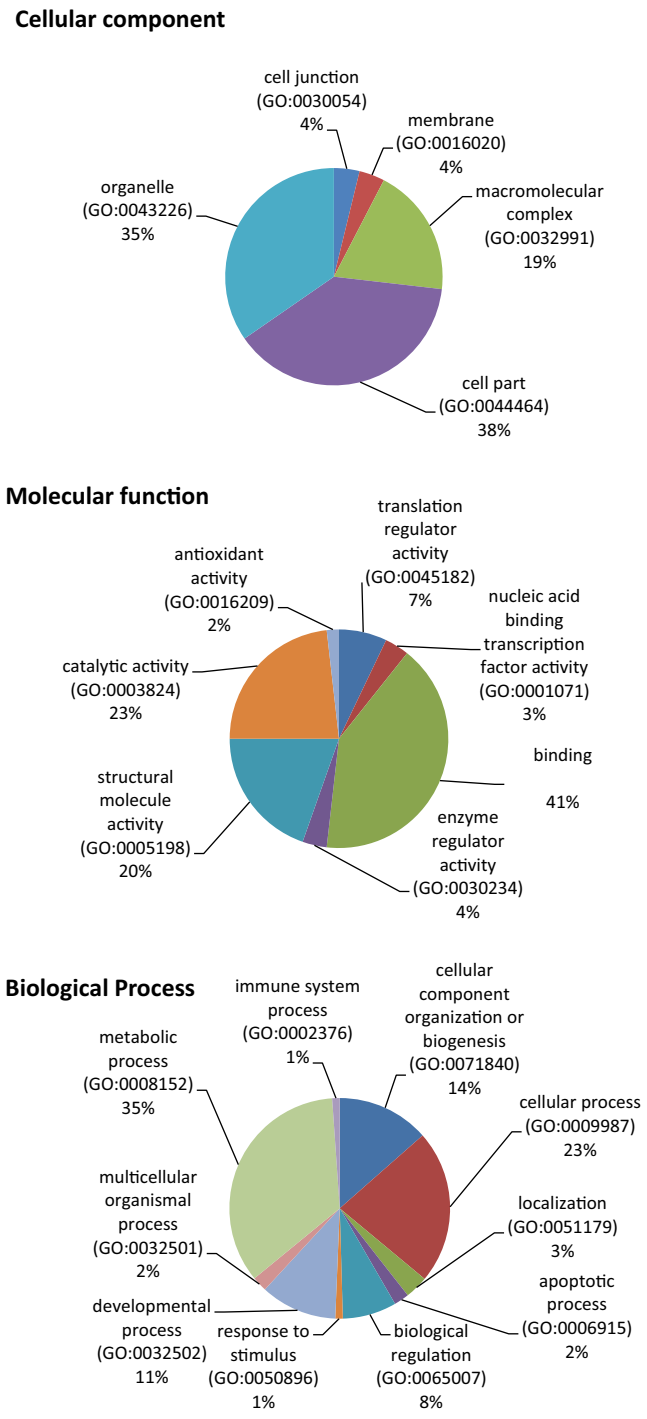

Fig. 1. Gene Ontology (GO) classification of identified proteins in DAOY medulloblastoma cells.

fragment mass "false", maximum retention time difference 0.5 min. Unspecific enzyme was set. Minimum and maximum peptide length was 5 and 144 residues, respectively, and peptide confidence was set to "high". Dynamic methionine oxidation (+15.99 Da) was set. Protein and peptide spectra matches were validated by using the Target Decoy PSM Validator that estimates the number of false positive identifications among all those found. The strict target false discovery rate (FDR) value was set to 0.01, while the relaxed FDR value was set to 0.05.

2.6. Functional clustering, Protein Classification and Protein-Protein Interaction analyses

All detected proteins were classified according to the Gene Ontology (GO) annotations, regarding molecular function, biologically process, and cellular component. Dataset processing was performed by the Protein ANalysis THrough Evolutionary Relationships (PANTHER) Classification System (version 9.0, http://www.pantherdb.org).

In order to determine the functional relationships among proteins, the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database of physical and functional interactions (version 10) was used employing the highest confidence (score 0.900) [10].

3. Results and discussion

DAOY cell lysates in acidic solution have been analyzed by HPLC-ESI-Orbitrap Elite-MS, in order to characterize the intact proteome by a top-down proteomic strategy. Table 1 lists the proteins and peptides identified in cell lysates after 24 h of starvation in serum-free medium. The corresponding Uniprot accession number, protein name, sequence coverage, and molecular mass data, along with the number and the amino acid sequence of the fragment peptides is included for each identified protein. Protein and peptide identification was accomplished by both software elaboration and manual inspection of MS/MS data (see materials and methods section for details).

Tandem MS software-based data analysis allowed identifying 47 proteins, most of them being disclosed through the characterization of peptide fragments. For some proteins, the number (n) of fragment peptides detected was high, in particular, for vimentin (MW 53.6 kDa, n = 16), peptidyl-prolyl *cis*-trans isomerase A (MW 18.0 kDa, n = 8), glyceraldehyde-3-phosphate-dehydrogenase (MW 36.0 kDa) and profilin-1 (MW 15.0 KDa) (both n = 6), heterogeneous nuclear ribonucleoprotein A2/B1 (MW 37.4 kDa, n = 5), elongation factor 1-alpha 1 (MW 50.1, n = 4), S100A11 (MW 11.7 kDa, n = 4) and thymosin beta-4 (MW 5.0 kDa, n = 3).

Given that sample treatment included a protease inhibitor cocktail, it may be reasonably assumed that these fragment peptides are either produced *in vivo* from parent proteins, with potential specific biological functions, or are released as a result of an extensive protease activity of DAOY cells. Few proteins, namely,

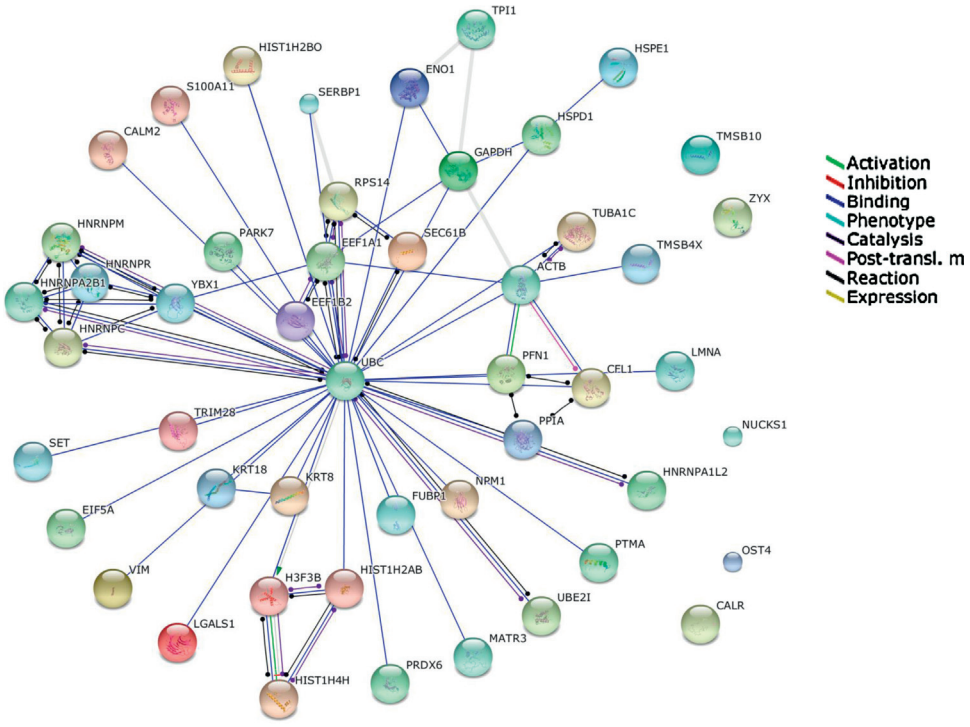

Fig. 2. Biological interaction network of proteins identified in DAOY cells. Figure produced by use of action view "Interacting" of STRING tool.

dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 4, thymosin beta-10 and beta-4, thymosin alpha-11, protein S100-A11, prothymosin alpha isoform 2, and ubiquitin, have been characterized entirely or with high sequence coverage. The manual inspection and annotation of MS^2 spectra identified 6 additional proteins and peptides. These included acetylated forms and isoforms: the isoform 2 of prothymosin alpha and its biologically active fragment 2–37 thymosin alpha-11, both N-terminal acetylated; the heterogeneous nuclear ribonucleoprotein K; the eukaryotic translation initiation factor 4H; the ubiquitin and the chaperonin 10-related protein. All the alpha- and beta-thymosins identified, listed in Table 1, along with the S100A11 protein, were N-terminally acetylated after Met1 removal. The isoform 2 of prothymosin alpha differs from isoform 1 in missing one glutamic acid residue (des-E40).

Table 2
List of the protein identified both in DAOY cells and medulloblastoma tumor tissue pool [11].

Accession[a]	Description
P06454	Thymosin alpha 11
P0CG48	Polyubiquitin-C
P06454-2	Prothymosin alpha isoform 2
P04908	Histone H2A type 1-B/E
P63313	Thymosin beta-10
P62328	Thymosin beta-4
P06733	Alpha-enolase
P62158	Calmodulin
P10809	60 kDa heat shock protein, mitochondrial
Q9BQE3	Tubulin alpha-1C chain
P40926	Malate dehydrogenase, mitochondrial
P62937	Peptidyl-prolyl *cis*-trans isomerase A
P60709	Actin, cytoplasmic 1
P60174	Triosephosphate isomerase
P22626	Heterogeneous nuclear ribonucleoproteins A2/B1
P04406	Glyceraldehyde-3-phosphate dehydrogenase

[a] Bold accessions refer to proteins identified also in medulloblastoma classic histotype [14].

It is noteworthy that 25 proteins (underlined accessions in Table 1) identified in this study have, to the best of our knowledge, never been described in previous proteomic studies of DAOY cells.

The GO classifications of the identified proteins based on cellular component, molecular function and biological processes, are reported in Fig. 1. The majority of them are components of organelles (35%), cell parts (38%) and macromolecular complexes (19%), and are mainly involved in binding (41%), catalytic (23%) and structural (20%) activities. Metabolic and cellular processes were the most prevalent annotations (see Fig. 1). In addition, the connections and functional interactions between the protein entries listed in Table 1, have been evaluated by the STRING search tool (Fig. 2). With the exception of calreticulin (CALR), thymosin β10 (TMSB10), zyxin (ZYX), dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 4 (OST4), and nuclear ubiquitous casein and cyclin-dependent kinase substrate 1 (NUCKS1), all the identified proteins appeared functionally interconnected, mainly through binding interactions.

The identification of alpha- and beta-thymosins in DAOY cells is particularly noteworthy. We have previously demonstrated the presence of this family of proteins and peptides in medulloblastoma tissues [11], and postulated a possible role as biomarkers of tumor aggressiveness. In particular, the overexpression of thymosin beta-4 and the exclusive presence of thymosin beta-10 and of alpha-thymosins, (including thymosin alpha-11) specified the malignant medulloblastoma tissue from the low grade pilocytic astrocytoma [11]. The finding of thymosin alpha-11 and thymosin beta-10 in the DAOY cell line could, therefore, confirm the importance of these peptides in characterizing the aggressiveness and high malignancy of this tumor.

Other proteins and peptides identified in DAOY cells have been previously characterized also in medulloblastoma tumor tissues [11–14]. Cofilin-1 and 60 kDa heat shock mitochondrial protein have been identified in anaplastic medulloblastoma, whereas the heterogeneous nuclear ribonucleoprotein k and calmodulin were found expressed in the classic and desmoplastic/nodular subtypes, respectively [12]. In particular, the heterogeneous nuclear

ribonucleoproteins resulted altered in MYC-amplified medulloblastoma tumors belonging to the molecular subgroup 3 [13]. The resulting alteration of the glycolytic metabolism may possibly explain the tumor aggressiveness and cell viability. Table 2 lists the species identified in medulloblastoma tissue pool by top-down proteomic approach in our previous paper [11]. Some of these proteins (accession in bold) have been also characterized in classic medulloblastoma histotype by 2DE-MALDI/TOF analysis [14].

The full list of proteins identified in DAOY cells (Table 1) were explored in DAOY cells cultured under the same experimental conditions and different time of starvation, namely 48 and 72 h. Out of the 53 proteins identified at 24 h, 37 were stably expressed in DAOY cells over time (displayed in Table 1 as bold-case accession number). The proteins were further characterized based on their peptide fragments, most of which were reproducibly detected at all time points after starvation (peptides in bold, Table 1). In particular, beta-thymosins and S100A11 retained the N-terminal acetylation PTM over the time course. As far as alpha-thymosins are concerned, only the alpha-11 peptide was conserved, whereas its parent protein, namely the prothymosin alpha isoform 2, was undetectable at 48 and 72 h.

These proteins, and particularly, their fragment peptides, were not influenced by culture time, indicating that they are associated with DAOY cells, regardless of starvation. These proteins/protein fragments could represent interesting clues for testing conditions/drugs able to perturb tumor growth using the DAOY cells as model.

4. Conclusions

Top-down proteomic analysis of a DAOY medulloblastoma cell line provides a new approach for unraveling the multifaceted features of the proteome of brain tumors, by both confirming previous findings and generating new information. Unlike the bottom-up approach, the top-down strategy has the advantage of being able to characterize intact proteins and peptides with their PTMs and proteoforms. The naturally occurring peptide fragments require further investigations, to clarify their correlation to the corresponding parent proteins and to functionally validate their biological functions. In this study, the presence of protein fragments may be the result of the activity of intracellular proteases, part of the so-called cancer “degradome” [15]. These may be either specific, i.e. functionally significant, or aspecific peptides.

Although some of the identified proteins have already been described in DAOY cells, the characterization of their naturally occurring fragment peptidome was not reported before. Our data, therefore, provide original hints towards the complete characterization of medulloblastoma molecular features.

The profiling of tumor cell cultures proteome/peptidome represents a valid tool to characterize tumors at molecular level and to support whole tissue studies. In particular, our data illustrate that top-down proteomic analysis is essential to distinguish protein isoforms, PTMs, naturally occurring biologically active peptides and protein fragments, which may all be crucial in tumor etiopathogenesis.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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