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FOCUS SESSION

A systematic view of pediatric medulloblastoma proteomics—current state of the field and future directions

Riccardo Serra[1] · Antonella Mangraviti[2]

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Abstract

Quantitative mass spectrometry (MS)–based approaches have allowed further characterization of medulloblastoma (MB) classification and clinical/biological behavior. By investigating protein expression, as well as the role of post-translational modifications in shaping cellular activity, novel avenues of research will clarify the current subgrouping, providing elements for tumor treatment—new molecular targets and signaling cascades—and introducing serum, urinary, and CSF markers of tumor growth and recurrence. We systematically searched and reviewed original research articles treating MB proteomics on PubMed. Reviews, opinion papers, and abstracts were excluded from the final work. A total of 30 novel articles treating the proteomic characterization of MB were included in our review. Research conducted on tissue samples, cell lines, CSF, and urine, as well as exosome and medullospheres, was considered, to picture a broad view of the different directions MS-based proteomic analysis is moving toward. In this review, we collect, summarize, and interpret the current literature on this topic. Significant progress has been achieved in the last decade in MB characterization, paving the way for further exploration of large biobanks of MB and other tissues that will allow a more systematic understanding of MB functioning and clinical progression.

Keywords Quantitative mass spectrometry · Medulloblastoma · Central nervous system

Introduction

Medulloblastoma, the most common pediatric tumor of the central nervous system (CNS), has been the object of extensive research and formidable advances in its molecular understanding. Despite this, medulloblastoma (MB) is still a leading cause of death for children under the age of 16 in most of the Western hemisphere, and vast discrepancies exist between the life expectancy of each subgroup. The current consensus recognizes four major molecular types, namely Wingless (WNT), Sonic hedgehog (Shh), group 3, and group 4 MB [1–5]. While relatively good prognosis can be achieved in WNT and group 4 tumors, Shh-driven (with mutated Tp53) and group 3 still retain a survival rate of 41% and 50% at 5 years, respectively. It is therefore not surprising that most of the research efforts in this field are pointed toward finding novel and radical approaches to first understand, and then treat, this malignancy.

Despite the growing body of genetic and transcriptomic data that support this classification and keep adding new evidence to each specific subgroup, the contribution of proteomic analysis has only partially impacted this malignancy so far. In recent times, a number of papers have begun to explain the role of proteomics and post-transcriptional modifications in shaping the behavior, aggressiveness, and sensitivity to treatments of MB [6, 7]. In the next years, the field will likely see a substantial growth in complexity and extent, with potential for substantial changes in MB classification and grouping. Furthermore, multi-omics approaches potentially will provide new markers of disease, both for diagnosis and for post-resection follow-up, and offer new targets for molecular therapies and pathway inhibitors.

This review aims at collecting and summarizing for the first time the existing literature in the field, offering a perspective on its contributions and likely future directions, with a special focus on the different branches and applications that stem from mass spectrometry and proteomic analysis.

✉ Riccardo Serra
rserra@som.umaryland.edu

[1] Department of Neurosurgery, University of Maryland - Baltimore, 22 St. Greene Street, Baltimore, MD 21201, USA

[2] Department of Neurosurgery, School of Medicine, Catholic University of the Sacred Heart, Rome, Italy

Methods

A systematic review of the literature was carried out to identify the published articles that focused on investigating the proteome and post-translational modifications (PTMs, among which phosphoproteomics has held a central role in the last years and in MB research) of MB tissue and patient-derived CSF and urine, through both bottom-up and top-down approaches (that is looking at either digested or intact entities retaining their PTMs). A PubMed search was conducted, and included "proteomics," "medulloblastoma," "phosphoprotemics," and "proteomic analysis." Abstracts were reviewed, only manuscripts presenting new research were included, and full texts were investigated and considered in the final analysis. Abstracts not associated to a published manuscript were excluded. Figure 1 and Table 1 summarize and outline the selection process used for this review.

A total of 30 studies that met these criteria were identified and considered for the final manuscript. Data from each of these papers was included in the review and interpreted in the "Discussion" section.

Results

The recent molecular subdivision of MB into four main molecular types, WNT-driven, Shh-driven, and groups 3 and 4, has inaugurated a new era in the understanding of this tumor's biological and clinical behavior, inspiring novel therapeutic strategies and spurring further investigation of its molecular drivers. Nonetheless, the genetic characterization of MB's DNA and mRNA has not been accompanied by a similar development on the proteomic counterpart, leaving several important questions on the behavior of MB cells unanswered [6, 7]. In the last years, a significant effort has been oriented toward understanding and describing the role of proteomics and phosphoproteomics in MB, and initial evidence suggest a pivotal role for this approach in directing future research and the quest for better therapies against this aggressive tumor of the CNS. In the sections, we present the available evidence obtained not only from tumor specimens, but also from patient-derived cell lines, exosomes, and CSC-secreted medullospheres, as well as the most recent attempts to characterize CSF and urinary markers of MB.

Proteomic and phosphoproteomic profiling

Recent advances in proteome quantification and detection have made possible the investigation of the proteomic and post-translational phosphoproteomic profiles of MB subgroups, allowing new research on the classic MB classification and integrating the current knowledge with post-transcriptional modifications/mechanisms of pathobiology (Fig. 2).

By analyzing Shh-driven, group 3, and group 4 MBs, with PTK (protein tyrosine kinase) and STK (serine/threonine kinase) PamChip arrays, Zomerman et al. identified two major signaling profiles, defined as protein-signaling clusters 1 and 2 (Fig. 3) [29]. Protein-signaling cluster 1 showed high mRNA expression of MYC or MYCN, as well as of Tp53, and dismal survival upon recurrence compared to Profile 2 ($p < 0.01$). To confirm these findings, assessment of RPE-1 cells with overexpression of MYC/MYCN/CCNE1/CDC25A and wild-type/mutated/null Tp53 was performed. These in vitro data showed that both MYC and MYCN overexpression induce a strong shift in peptide phosphorylation profile compared to empty vector cells, amplified by Tp53 loss. Importantly, only 2 tumors had MYC amplification in their cohort, and the higher Tp53 mRNA expression likely drove the pro-oncogenic profile shift. Protein-signaling cluster 2 on the other hand is enriched in apoptotic signaling, DNA damage/p53-mediated response, and neuronal signaling, with marked reduction in functional p53 protein. The authors hypothesized a role for decreased p53 behind the accumulation of precociously dedifferentiated neurons (secondary to the impairment of apoptosis) and MB origin. In this cluster, there may be a role for p53 enhancers, such as Nutlin-3, as new targets of therapy.

While Shh-driven and group 3 tumors mainly exhibited a protein-signaling profile of type 1, group 4 tumors were mostly characterized by DNA damage response and neuronal/apoptotic signaling, resembling protein-signaling cluster 2.

Another important contribution by Forget et al. classified 3892 proteins and 4950 phosphosites via peptide fractioning and phosphopeptide enrichment, revealing several inconsistencies with the RNA seq and DNA methylation classifications [11]. In particular, group 4 tumors exhibited a completely different phosphorylation profile from the other subgroups. Similarity network fusion (SNF) analysis efficiently segregated G3 from G4 tumors, better than RNA seq and DNA methylation, highlighting distinct post-transcriptional regulation in MB subgroups to control the final protein output level.

G4 signaling RTK activation is the hallmark feature of this subgroup, both as protein/mRNA ratio and at absolute levels. Global tyrosine phosphorylation was increased in this group compared to G3, validating the activation of this signaling network, and downstream mediators (PI3K, MAPK, and mTOR) were also increased.

- ERBB4 and its ligand NRG2 were the most expressed kinases in G4, as confirmed by mRNA analysis. MAPK and PI3K-AKT were also hyper-expressed in G4 tumors, as well as BRAF, CRAF, ERK1/ERK2, and TSC1/TSC2.

Table 1 List of the final papers selected for the review. First author's name, article's title, journal, methodology used, and main findings are presented in the table

Authors	Title	Journal	Methods	Findings
Archer et al. [8]	Proteomics, Post-translational Modifications, and Integrative Analyses Reveal Molecular Heterogeneity within Medulloblastoma Subgroups	Cancer Cell, 2018	Quantitative profiling of proteomes and phosphoproteomes of 45 samples	Distinct SHH pathways, MYC mutations in group 3
Gu et al. [9]	Proteomic profiling of isogenic primary and metastatic medulloblastoma cell lines reveals differential expression of key metastatic factors	J Proteomics, 2017	Quantitative profiling of CHLA-01-MED and CHLA-01R-MED proteomes	~ 1400 distinct proteins between the primary and metastatic lines
Rivero-Hinojosa et al. [10]	Proteomic analysis of Medulloblastoma reveals functional biology with translational potential	Acta Neuropathol Commun, 2018	SILAC quantitative proteomics of MB and control cerebellum	EIF4F cap-dependent translation as a novel druggable pathway
Forget et al. [11]	Aberrant ERBB4-SRC Signaling as a Hallmark of Group 4 Medulloblastoma Revealed by Integrative Phosphoproteomic Profiling	Cancer Cell, 2018	Quantitative (phospho)-proteomics of primary MBs	Group 4 aberrant ERBB4-SRC signaling
Staal et al. [12]	Proteomic profiling of high risk medulloblastoma reveals functional biology	Oncotarget, 2015	Quantitative profiling of group 3 proteomes	MYC-amplified tumors are related to glycolytic metabolic pathways.
Purzner et al. [13]	Developmental phosphoproteomics identifies the kinase CK2 as a driver of Hedgehog signaling and a therapeutic target in medulloblastoma	Sci Signal, 2018	Phosphoproteomics of murine GNPs	CK2 stabilizes GLI1 and Shh signaling, inhibition with CX-4945 reduces MB growth.
Ronci et al. [14]	Proteomic analysis of human Sonic Hedgehog (SHH) medulloblastoma stem-like cells	Mol Biosyst, 2015	Proteomic analysis of SHH-MB SLCs pre and post-retinoic acid, ingenuity pathway analysis	68 modulated proteins between SLCs and differentiated cells, PI3K/Akt/NF-κB activation
Anagnostopoulos et al. [15]	Proteomic studies of pediatric medulloblastoma tumors with 17p deletion	J Proteom Res, 2015	Quantitative profiling of MB vs normal cerebellar tissue	PI3K/mTOR and IGF signaling upregulation
Tsangaris et al. [16]	Molecular Proteomic Characterization of a Pediatric Medulloblastoma Xenograft	Cancer Genomics Proteomic, 2017	MALDI-TOF MS analysis of subcutaneous mice xenografts	350 single-gene products, reported in a MBLX database
Spiombi et al. [17]	KCTD15 inhibits the Hedgehog pathway in Medulloblastoma cells by increasing protein levels of the oncosuppressor KCASH2	Oncogenesis, 2019	MALDI-TOF MS analysis of HEK293T	KCASH2 loss causes Hh hyperactivation. KCTD15 stabilizes KCASH2, reducing cell proliferation.
Kaid et al. [18]	Proteome and miRNome profiling of microvesicles derived from medulloblastoma cell lines with stem-like properties reveals biomarkers of poor prognosis	Brain Res, 2020	DAOY, CHLA-01-MED, D283-MED, and USP13-MED microvesicular proteome analysis	ERK, PI3K/AKT/mTOR, EGF/EGFR, and stem cell self-renewal signaling
Higdon et al. [19]	Integrated Proteomic and Transcriptomic-Based Approaches to Identifying Signature Biomarkers and Pathways for Elucidation of Daoy and UW228 Subtypes	Proteomes, 2017	Total transcriptome and proteome of DAOY and UW228 cells	Differential expression of adhesion, cytoskeletal and signaling molecules, enrichment of WNT/Shh pathways
Zanini et al. [20]	Medullospheres from DAOY, UW228 and ONS-76 cells: increased stem cell population and proteomic modifications	PLOS One, 2013	MALDI-TOF analysis of DAOY, UW228, and ONS-76 medullospheres	Nucleophosmin and SOX-2 identified in CSCs
Martelli et al. [21]	Top-down proteomic characterization of DAOY medulloblastoma tumor cell line	EuPA Open Proteom, 2016	LC-MS top-down approach of DAOY cells	Thymosins and other pro-inflammatory peptides
Peyrl et al. [22]	Protein profiles of medulloblastoma cell lines DAOY and D283: identification of tumor-related proteins and principles	Proteomics, 2003	2D electrophoresis of DAOY and D283 cells	Ded antiapoptotic protein and hypothetical proteins were identified in both lines.
Zanini et al. [23]	Analysis of different medulloblastoma histotypes by two-dimensional gel and MALDI-TOF	Childs Nerv Syst, 2011	2D gel electrophoresis of 8 MB samples	Identification of type-specific proteins with limited overlapping between subgroups
Rajagopal et al. [24]	Proteomic profiling of cerebrospinal fluid identifies prostaglandin D2 synthase as a putative biomarker for pediatric medulloblastoma: A pediatric brain tumor consortium study	Proteomics, 2011	MALDI-TOF TOF MS analysis of 33 MBs and 25 controls	Decrease levels of PGD2S in tumor samples and CSF compared to controls
Xu et al. [25]	Loss of Pin1 Suppresses Hedgehog-Driven Medulloblastoma Tumorigenesis	Neoplasia, 2017	Protein co-immunoprecipitations in a MB line	PIN1 promotes GLI1 protein abundance, with consequent Hh activation.
Bisaro et al. [26]	Proteomic analysis of extracellular vesicles from medullospheres reveals a role for iron	Mol Cell Ther, 2015	Proteomic analysis of stabilized medullospheres from MB lines	Elevated iron carrier proteins in CSC-derived MS; iron-chelators reduce MS number/-dimensions.

Table 1 (continued)

Authors	Title	Journal	Methods	Findings
	in the cancer progression of medulloblastoma			
Gotschel et al. [27]	Synergism between Hedgehog-GLI and EGFR signaling in Hedgehog-responsive human medulloblastoma cells induces downregulation of canonical Hedgehog-target genes and stabilized expression of GLI1	Plos One, 2013	Analysis of Daoy cells after HH/EGF co-treatment	GLI1, PTCH, and HHIP downregulation. EGFR and Hh synergy can cause a HH/GLI profile switch to target gene profile.
Narayan et al. [28]	Proteomic profiling of medulloblastoma reveals novel proteins differentially expressed within each molecular subgroup	Clin Neurol Neurosurg, 2020	Proteomic profiling of 6 MBs and 6 controls	Upregulation of PMEL/FBN2 in WNT MB, SYNGR2 in SHH MB, and GFAP/IMPG2/MAGEA10 in non WNT/Non SHH MB
Zomerman et al. [29]	Identification of Two Protein-Signaling States Delineating Transcriptionally Heterogeneous Human Medulloblastoma	Cell Rep, 2018	Phosphoproteomic analysis of 50 MBs	MYC-like profile with expression of cell cycle, GPCR, and protein signaling—rapid death at recurrence; protein-signaling profile 2 with apoptosis, neuronal, and DNA damage signaling—frequent metastases at diagnosis
Epple et al. [30]	Medulloblastoma exosome proteomics yield functional roles for extracellular vesicles	PLOS One, 2012	Tandem mass spectrometry analysis of D283MED and DAOY exosomes	HNF4A role for tumor suppression, and pathway targeting for future therapies
Low et al. [31]	Cerebrospinal fluid cytokines in metastatic group 3 and 4 medulloblastoma	BMC Cancer, 2020	Proteomic array CSF analysis from metastatic and non-MB	Higher CCL2 expression in metastatic group 3/4 patients
Zanini et al. [32]	Immunohistochemical and proteomic profile of melanotic medulloblastoma	Pediatr Blood Cancer, 2009	Study of protein profiles in melanotic versus non-melanotic regions	Melanocytic-associated antigens and epidermal autoantigen 450K in melanotic nodules
Reichl et al. [33]	Determination of a Tumor-Promoting Microenvironment in Recurrent Medulloblastoma: A Multi-Omics Study of Cerebrospinal Fluid	Cancers, 2020	Mass spectrometry–based multi-omics study of CSF from MB vs control patients	Upregulation of FSTL5, ART3, and FMOD, with potential for use as MB markers
Hao et al. [34]	Urinary protein biomarkers for pediatric medulloblastoma	J Proteomics, 2020	Tandem mass tag of urinary samples from pre- and post-op MB. Markers were validated in 112 samples.	CADH1, FGFR4, and FIBB could be used as a model to identify MB patients from controls with an AUC of 0.973.
Scheidt et al. [35]	Phosphoproteomics of short-term hedgehog signaling in human medulloblastoma cells	Cell Commun Signal, 2020	Quantitative phosphoproteomics after short-term activation and inhibition of Shh MB	ERK/MAPK, protein kinase A, and mTOR differentially regulated after short-term treatment. Phosphorylation of mediators of the Hh pathway—SMO, SUFU, GLI2, and GLI3—after 15 min
Gruber-Olipitz et al. [36]	Synthesis, chaperoning, and metabolism of proteins are regulated by NT-3/TrkC signaling in the medulloblastoma cell line DAOY	J Proteome Res, 2008	MALDI-TOF-TOF analysis of DAOY cells after TrkC activation	Translational, splicing, processing, chaperone, protein handling, and metabolism depend on neurotrophin-3-induced TrkC activation.
Azizi et al. [37]	Identification of c-myc-dependent proteins in the medulloblastoma cell line D425Med	Amino Acids, 2012	Mass-spec analysis of D425med with knocked-down c-myc	Signaling, protein synthesis, metabolism, and ER function are affected by c-myc inhibition.

- GPCR was enriched and activated in G4 tumors, as well as its downstream effectors CDC42, RAC1, PAK2, ROCK2, and BORG.
- SRC, a key regulator of RTK signaling, was elevated in G4 tumors.

The nuclear transitory zone seems to be the area of origin for G4 tumors, as confirmed with immunohistochemistry and in utero electroporation gene transfer. SRC activation produced G4 tumors in posterior cerebellum and dorsal hindbrain, with a radically different profile from that of MYC-induced G3 tumors. This finding confirms SRC as a potential target for new therapies.

G3 signaling These tumors were significantly more enriched in translation-related functions (eIF complex and mRNA processing/metabolism proteins); in addition, 3 protein complexes related to the ubiquitin proteasome complex (UPC) were identified (Skp, Cullin—with neddylation of DCUN1D1, F box containing complex—SCF) in G4.RBP (RNA binding proteins), and nucleolin (NCL) and cold inducible RNA BP (CIRBP) were found at higher levels in G3

tumors, as well as a larger pool of non-phosphorylated and active EIF4EBP1. Higher levels of eIF complex–related proteins (EIF2, EIF4G, EIF4A) were found in G3 compared to G4 tumors, suggesting a potential pathobiological role for these mediators.

Interestingly, a significantly lower correlation between mRNA products and protein expression was found in group 3 and 4 tumors, highlighting once more the importance of post-transcriptional mechanisms of protein modification and expression in MB pathobiology.

Archer et al. found that RNA and protein stability, translational regulation, and signaling cascades differ among Shh tumors, and divided them into ShhA and ShhB MBs. Furthermore, while ShhA tumors had a predominance of proteins associated with mRNA processing/splicing/transcription, as well as with the MYC pathway, chromatin remodeling, and DNA repair, ShhB MB is usually characterized by neuronal and neuro-transmitter-like activity, with higher expression of CD47, glutamate, calcium, and MAPK/ERK signaling [8].

Several other studies identified specific markers or proteins as potentially responsible for MB aggressiveness and clinical behavior, both in patient-derived samples and in commercial tumor cell lines.

Rajagopal et al. hypothesized a role for PG2D synthase as a biomarker of MB, noting a decrease in 6 isoforms of PG2D synthase in MB tissue and CSF [24]. However, their levels were not statistically different between totally and partially resected tumors, probably secondary to the long turnover of this protein. Purzner et al. provided important evidence that CK2 substrates are usually more phosphorylated at p7 in GNP cells compared to post-mitotic p14, suggesting a role for this mediator in the HedgeHog signaling cascade [13]. To further reinforce this hypothesis, in vitro evidence was gathered from $Ptch^{+/-}$, $Tp53^{-/-}$, and SmoD477G mouse MB cells, where Vismodegib-resistant cells were sensitive to Cx-4945, a selective CK-2 inhibitor. Interestingly, patients with Shh-driven tumors expressing lower levels of CK-2 mRNA showed significantly longer survival than those with higher mRNA burden. Scheidt et al. contributed to the understanding of phosphoproteomics in HH signaling by studying the short-term responses to its inhibition and activation [35]. Phosphorylation was found to have a central role in directing ciliary assembly, cell trafficking, and signal transduction, particularly in ERK/MAPK, PK-A, and mTor cascades. Phosphorylation of SMO, SUFU, GLI-2, and 3 was observed after longer treatment; exposure to Vismodegib determined inhibition of casein kinase 2A1, while SAG treatment activated aurora kinase A signaling. Additional evidence on the functioning of the HH cascade was presented by Spiombi et al. In their work, the role of the oncosuppressor KCASH2 (part of the KCASH family and a known regulator of HH signaling in cerebellar growth and differentiation) was investigated through MALDI-TOF analysis of HEK293T cells [17]. By negatively regulating Gli1 signaling, a mediator of HH activity that increases the expression of cyclin-D2 and N-myc, KCASH-2 is essential in controlling cerebellar and MB growth and malignancy. When this protein is missing, an

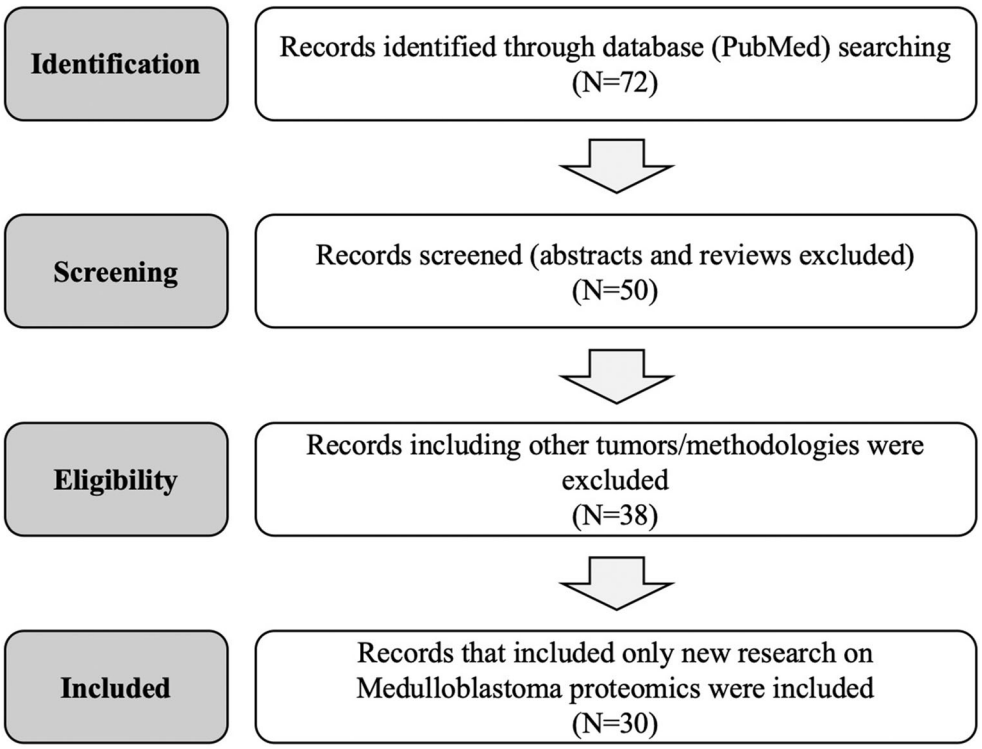

Fig. 1 Flow diagram of the systematic research and selection process used to identify the final papers used in this review

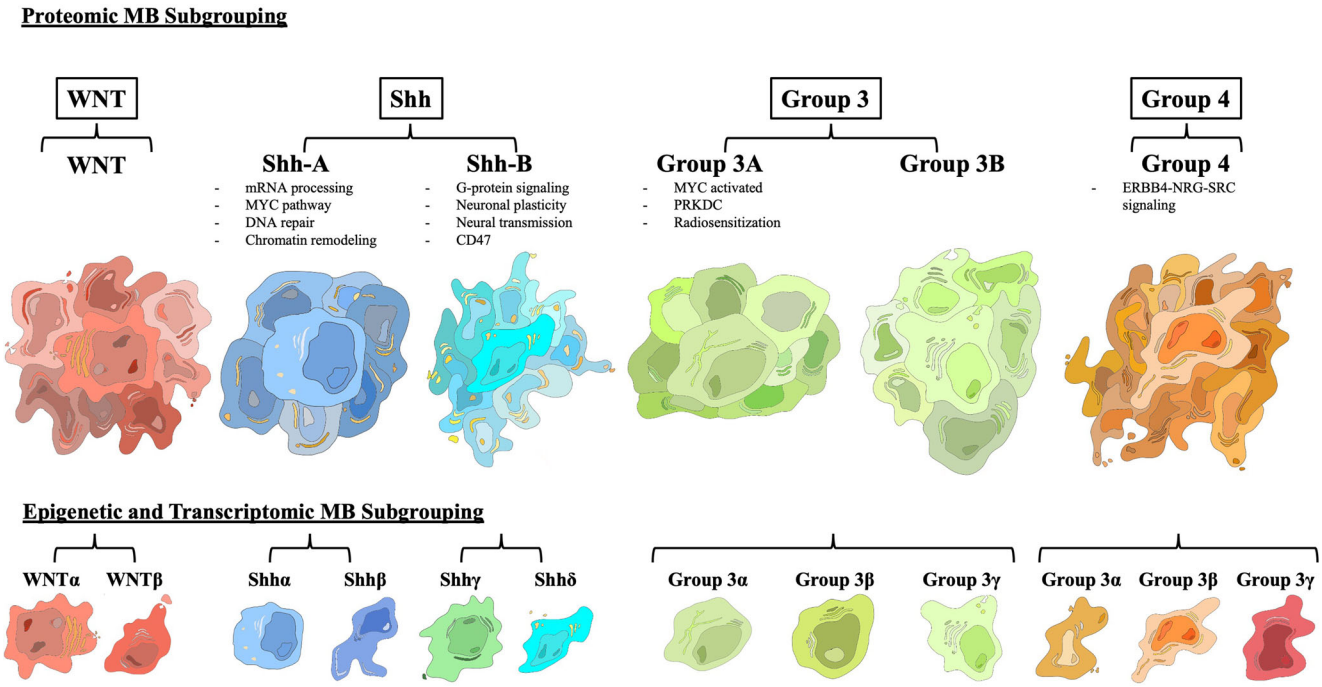

Fig. 2 Novel subgroups identified by Zomerman et al., Forget et al., and Archer et al. through proteomic and phosphoproteomic analysis of MB samples and cell lines. At the bottom, the classic genomic- and transcriptomic-based subgroups

uncontrolled upregulation of this pathway takes place, leading to faster and more aggressive tumor growth and HH activation.Martelli et al. attempted to characterized DAOY cells with an LC-MS top-down approach, looking at intact and native proteins and peptides, disclosing the potential role of post-translational modifications on cell behavior [21].

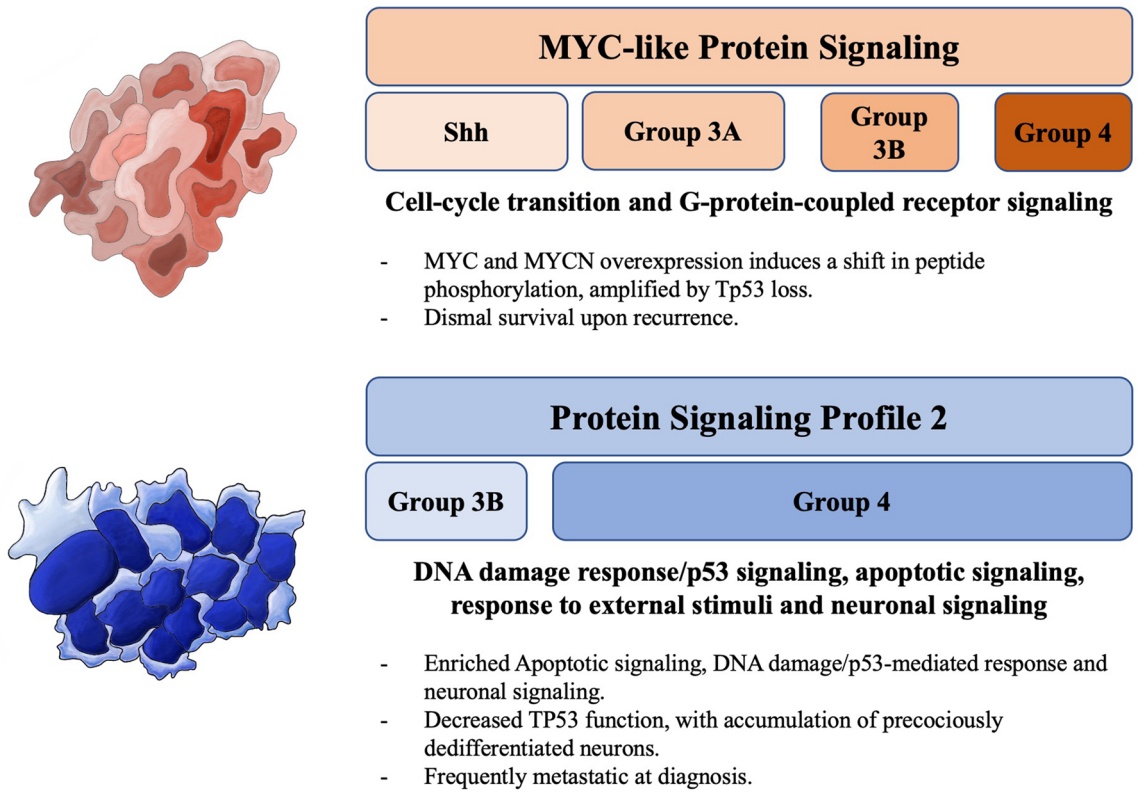

Fig. 3 Protein-signaling profiles identified by Zomerman et al. through PTS and STK phosphorylation profiling

Importantly, 25 new proteins were identified and classified through Gene Ontology (GO). Most of these were indeed related to biological processes (i.e., metabolic processes, apoptosis, immune reaction), molecular functions (i.e., protein translation, nucleic acid binding, enzymatic regulation), or served as cellular components in MB cells (i.e., membrane, organelles, cell junctions). Alpha- and beta-thymosins (specifically, alpha-11, beta-4, and beta-10) were identified using a top-down proteomic approach, suggesting a potential role for these markers of tumor aggressiveness in MB pathobiology, similarly to what was found in pilocytic astrocytoma and craniopharyngioma [38–41].

DAOY and UW228 gene ontology was also classified by Higdon et al., uncovering that while the first line has about 2630 unique peptides, the second was limited to only 1235. Interestingly, 1102 of these were shared between the two lines, leaving only 133 unique sequences to UW228 cells and 1528 to DAOY. While DAOY-associated proteins were mostly involved in signal transduction (EGFR, CRK), cell adhesion (JUP, TGFBI), stress resistance (HSP90AA1, HSP90AB1), tumor invasion/metastasis (TNC), and DNA binding/repair (MSH2, MSH6, TOP2A), UW228 proteome was characterized by higher representation of proteins related to cell adhesion (POSTN, MCAM, L1CAM), cytoskeletal organization (LASP1), stress resistance (CRYAB), cell signaling (DAB2, CSPG4), and tumor suppression (BASP1, UTRN). These differences partially explain the more aggressive behavior of DAOY, but also underline a substantial genetic and proteomic difference between lines comprised in different groups.

Xu et al. and Gotschel et al. both provided important evidence on the role of GLI activation for Shh tumors. Both EGFR-Hh synergy and PIN1 seem to hold a central role in determining the Hh/GLI profile switch, activating the Hh cascade in MB.

Exosome proteomics

Exosomes, with a diameter ranging from 30 and 100 nM, are particles derived from the endosomal system released during multivesicular body (MVB) formation. Their main roles are thought to be transport of toxic substances, as well as transfer of genetic information and viral movement. Furthermore, research in the past years has highlighted a potential involvement of exosomes in tumor dissemination, cell proliferation, and chemotherapy resistance, as well as in the immune response to malignant cells. Growing evidence is trying to conjugate the MB phenotype with the proteomic and signaling expression of these microscopic bodies.

Epple et al. by using pathway analysis demonstrated that chaperons (HSP/HSC70, HSP 90, 60, and 27), hemopexin, and ERBB2 (Her2/Neu cascade) are all preferentially expressed in exosomes, suggesting a role for these bodies in MB biology [30]. Interestingly, when exosomes were used as chemo-attractants in DAOY and UW228 cells, tumor cell migration was increased in a concentration-dependent fashion, activating also cell proliferation and stimulating interferon-γ secretion in T cells. Finally, hepatocyte nuclear factor 4 alpha (HNF4A) was characterized in exosomes and identified as a tumor suppressor and cell differentiator in group 3 line D283MED, while its inhibition with the anti-diabetogenic drug MEDICA16 showed higher growth rates in a concentration-dependent fashion.

Proteomics of MB medullospheres (MS)

Zanini et al., through Maldi-TOF analysis of the proteomic pattern in DAOY, UW228, and ONS76 cells, revealed a number of key differences between adherent and sphere-like cells [32]. Seventy-seven proteins were differentially expressed between the two states, particularly in UW228 and DAOY lines, where 10 and 9 proteins differed in medullospheres, respectively. Among them, nucleophosmin, cofilin-1, stathmin, calpastatin, and Poc1—all markers of anaplastic behavior and drug resistance—were differentially expressed in suspended cells. Bisano et al. highlighted a pivotal role for iron in DAOY, UW228, and ONS76 MS maintenance [26]. Hemopexin and serotransferrin were found to be increased in MB cells, while Fe^{++}-chelators permeable to the cell membrane were able to decrease the number and size of MS, suggesting a potential avenue for future treatment. Finally, Kaid et al., by investigating DAOY, CHLA-01-MED, D283-MED, and USP13-MED microvesicles with proteome analysis, highlighted once more the preponderance of ERK, PI3K/AKT/mTOR, EGF/EGFR, and stem cell self-renewal signaling in these bodies, suggesting a potential role for MB aggressiveness.

Urinary markers of MB

Urinary and other systemic markers of pathology have attracted physicians and scientists over the years for their accessibility and limited invasiveness. Nonetheless, the need for reliable and affordable proteins or metabolites has made this quest extremely challenging, especially for those diseases that do not have a high prevalence in the pediatric populace. Hao et al. therefore importantly contributed to this field by introducing the first proteomic-based approach to identifying MB in patients [34]. By using a two-step approach, a "discovery stage" first and a "validation stage" at a later point, and four groups of patients (pre- and post-resection MB patients, benign brain disease control and healthy control patients), they were able to identify 114 differential proteins. Seventeen potential biomarkers were validated through parallel reaction monitoring (PRM), and a panel of CADH1, FGFR4, and

FIBB showed that MB and control patients could be discriminated with an AUC of 0.973, in a blinded way.

CSF markers of MB

Disease dissemination in the CSF compartment is a common and serious complication of MB natural history, especially in group 3 and 4 tumors. To better understand the tumor microenvironment and the role of inflammation in shaping its behavior, Low et al. attempted to describe the cytokine profile in a cohort of group 3 and 4 patients, by proteomic array analysis [31]. Interestingly, higher expression of CCL-2 was detected in the CSF of patients with metastatic disease. Furthermore, in a subgroup of group 3 MB with MYC amplification and metastatic/delayed metastatic MB, the authors found higher levels of CXCL1, IL-6, and IL-8 at the initial presentation, and that MYC amplification was a pivotal factor in determining cytokine expression in this group of tumors.

Reichl et al. also looked at the tumor microenvironment through CSF analysis, finding a prevalence of anti-inflammatory and tumor-promoting proteins and markers (FSTL5, ART, FMOD), with enzymes typically associated with conditions of tissue hypoxia (ADAMTS1, GAP43, GPR37) [33]. For the authors, these conditions promote a vicious cycle of hypoxia, tumor growth, and de-differentiation into cancer stem-like cells, probably driven by autophagy and a metabolic shift to beta-oxidation.

Discussion

Genomic, transcriptomic, and now proteomic approaches have revolutionized the understanding and classification of the most common pediatric brain tumor, MB, over the last two decades.

While the study of gene mutations and expression have importantly contributed to the definition of this tumor into the current consensus subgroups, a downstream analysis of its developmental pathways and proteomic profiles can provide pivotal information on how the genome, epigenome, and transcriptome interconnect to determine protein expression and eventually cell behavior.

In recent years, a number of studies in this nascent field revealed tangible genetic, transcriptional, and proteomic discrepancies, with notable shifts in DNA, mRNA, and protein ratios. While somewhat expected, these findings importantly suggest that the current subgrouping and tumor characterization is likely partial and incomplete, and that gene mutations and mRNA expression do not always coincide with the actual protein levels. A new, more accurate and complete classification of MB could therefore stem from the proteomic analysis of previously investigated and new cohorts of patients. This feat would not only offer a more accurate understanding of the biological behavior of MB subtypes, but also provide new targets of treatment for MB, opening a new era of patient- and pathway-centered research able to tailor treatments to the specific tumor biology.

Proteomic subgroup stratification

The current classification of MB is mainly based on DNA and mRNA sequencing, subgrouping tumors on their genetic signature at a pre-translational level. Although this method has deeply changed our approach to MB biology and treatment, several critical questions still remained unanswered by genomic and transcriptomic techniques.

A series of papers, most notably those by Archer et al., Forget et al., and Zomerman et al., have highlighted the discrepancies in tumor subgrouping of the current consensus [8, 11, 29].

Zomerman et al. demonstrated the existence of two separate signaling states, one characterized by MYC-like signaling, and a second with hyper-expressed protein-signaling. While both are associated with clinical prognosis and different biological behaviors, the uncovering of proteomic signatures that transcend the classical molecular subtyping clearly marks a breaking point with the current consensus, and opens to new potential proteome-based subgroups.

Similarly, Archer et al. divided Shh into ShhA and ShhB, and group 3 in 3A and 3B tumors based on post-transcriptional and post-translational modifications. Forget et al. identified subgroup-specific translational signatures in group 3 and 4 MBs, at the same time confirming the existing classification and unraveling a few inconsistencies between mRNA and genetic data, and their proteomic counterpart.

By adding another layer of molecular characterization, mass spectrometry could potentially uncover in a systematic, rapid, and highly reliable manner, the cellular behavior of MB, and its changes after treatment. A path toward widespread MS analysis for MB study and follow-up cannot overlook an initial fundamental step: a MS-based proteome map of normal cerebellar tissue and precursor elements (GNPs, GluCNs, LRL progenitors, and group 3 progenitors) should in fact be obtained to allow precise and unbiased data-dependent acquisition (DDA) of the different proteoforms. Only then, by either DDA or DIA (data-independent acquisition), large free-from-hypothesis, unbiased, and all-encompassing analyses of the MB proteome will be possible. In addition, multi-institutional large-scale international studies might be an effective way to collect and characterize a sufficient number of samples to translate the proteomic findings to drug and biomarker development.

Ultimately, a significant fraction of the studies presented here relied on existing patient-derived cell lines, an inexpensive instrument widely used in basic and translational research. However, while this methodology still has a pivotal

role in drug development and pre-clinical testing, its applications are limited in the multi-omics era. Remarkably, these lines represent only a fraction of the biological variability seen in nature, and while their study has value for internal line validation and as proof-of-principle investigation of a tumor's proteomics, the data extracted from them has limited general translatability to MB.

In the future, a patient- and sample-centered approach, in connection with the other -omics branches, appears therefore to be the only valid and scientifically sound approach to MB characterization.

Biomarkers for MB diagnosis/recurrence/metastasis

Mass spectrometry for characterization of MB proteomic profile has opened a new era for the discovery and validation of novel biomarkers of diagnosis and disease progression. By simultaneously analyzing and processing thousands of peptides and proteins at the same time, mass spectrometry and protein databases are able to quickly identify those peptides that more likely related to a specific subgroup of MB, and more importantly have the sensitivity and specificity to understand if the tumor is present, progressing, recurring, or metastasizing. Hao et al., Low et al., and Reichl et al. importantly demonstrated how MS and proteomics can efficiently join forces to rapidly and efficiently screen thousands of proteins, a task that in the future will likely be taken on by machine learning (ML) and artificial intelligence (AI) [31, 33, 34].

Despite the preliminary state of this field, the future will likely see larger cohort of patients followed longitudinally, whose tumors will be characterized at different levels by means of DNA and mRNA sequencing, in association with proteomic analysis of their proteomic and post-translational modifications spectra. Validation of these markers, in healthy controls and MB patients—both pre- and post-op—will play a fundamental role in establishing them as potentially helpful additions to current standard practices.

Investigation of new targets of treatment

One of the most powerful applications of proteomic research lies in the clinical translatability of its discoveries, and in particular in the direct connection between protein expression and target modulation. While DNA and mRNA analysis and stratification does not directly correlate with the tissue level of each gene, spectra analysis provides a precise estimate of the single protein expression. Moreover, this type of analysis bypasses the filter of post-translational modifications, providing a high-fidelity representation of a tumor's biology and phosphoproteome.

As a direct example of its power and direct translatability to clinical use, some of the kinases identified by Archer et al., such as GSK3B, CDK5, and PRKDC, as well as the mediators of the ERBB cascade investigated by Forget et al., might be useful targets for future treatments [8]. Other examples were provided by Rivero-Hinojosa et al. that, by comparing MB and normal cerebellar tissue, suggested EIF4F cap-dependent translation as a novel druggable pathway. Alternatively, also MYC and MYCN activation by post-translational modification of their sites could constitute an interesting tool for fine tuning their molecular signaling and reduce the biological aggressiveness of MB.

Importantly, the integration of proteomic analysis with new instruments such as CRISPR-Cas9 could provide additional depth to this approach. By specifically modifying a certain mediator in a signaling cascade, researchers will be able to reproduce and study in a controlled way those pathways that are thought to be responsible for the MB phenotype.

Conclusions

Epigenomics, transcriptomics, and proteomics have established themselves as the future of tumor research and translational investigation. Despite the latter field being in its early infancy, and the current lack of large cohorts of patients studied with an integrated approach, a more systematic, organized, and multi-level strategy for tumor biology and treatment investigation will likely take hold in the future.

Proteomic analysis in particular, both for the growing datasets and its vast practical applications, will likely play a pivotal role not only in tumor characterization and subgrouping, but also in identifying potential targets for treatment, and finding CSF and serum biomarker for tumor diagnosis and recurrence. These tasks, especially if developed concurrently and in concomitance with a multi-omic investigation of DNA mutations and expression in large multi-center prospective cohorts, are likely to uncover many of the still-obscure aspects of MB. Potentially, this could bring about a change in perspective as important as that witnessed in 2012 with the birth of the current consensus [1], opening new avenues for the cure of this aggressive malignancy.

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Compliance with ethical standards

Conflict of interest Authors have no conflicts of interest.

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