**Pediatric Ependymoma: A Proteomics Perspective**

GEORGE TH. TSANGARIS\(^1\), CHRISSA PAPATHANASIOU\(^2\), PANAGIOTIS G. ADAMOPoulos\(^3\), ANDREAS SCORILAS\(^4\), CONSTANTINOS E. VORGIAS\(^6\), NEOFYTOS PRODRouMou\(^3\), FOTEIINI TZORTZATOU STATHOPOULOu\(^2\), DIMITRIOS J. STRAVOPODIS\(^3\) and ATHANASIOS K. ANAGNOSTOPOULOS\(^1\)

\(^1\)Proteomics Research Unit, Biomedical Research Foundation of the Academy of Athens, Athens, Greece;  
\(^2\)Hematology/Oncology Unit, First Department of Pediatrics, University of Athens, Agia Sophia Children’s Hospital, Athens, Greece;  
\(^3\)Department of Neurosurgery, Agia Sophia Children’s Hospital, Athens, Greece;  
\(^4\)Department of Biochemistry and Molecular Biology, Faculty of Biology, School of Sciences, National and Kapodistrian University of Athens, Athens, Greece;  
\(^5\)Department of Cell Biology and Biophysics, Faculty of Biology, School of Sciences, National and Kapodistrian University of Athens, Athens, Greece

**Abstract.** Background/Aim: Proteomics based on high-resolution mass spectrometry (MS) is the tool of choice for the analysis of protein presence, modifications and interactions, with increasing emphasis on the examination of tumor tissues. Application of MS-based proteomics offers a detailed picture of tumor tissue characteristics, facilitating the appreciation of different tumor entities, whilst providing reliable and fast results for therapeutic marker targeting and prognostic factor assessment. Through use of the high analytical resolution of nano-high-pressure liquid chromatography (nanoHPLC) and the high resolution of an Orbitrap Elite mass spectrometer, the present study aimed to provide knowledge on the proteome of the generally unknown entity of pediatric ependymal tumors. Materials and Methods: Ten resected specimens of childhood ependymoma were analyzed through a one-dimensional (1D) nanoLC-MS/MS approach. Method optimization steps were undertaken for both the sample preparation/protein extraction procedure and LC parameters, aiming to achieve the highest possible identification rates. Results: Following method optimization, each nanoLC-MS/MS run resulted in identification of more than 5,000 proteins and more than 25,000 peptides for every analyzed sample, thus detailing the greater part of the ependymoma proteome. Identified proteins were found to spread throughout all known tumor categories regarding their molecular function and subcellular localization. Conclusion: Through the proposed nanoLC-MS/MS method herein we report, for the first time, the ependymoma proteome database. A large number of similarities regarding proteome content are revealed compared to other two pediatric brain tumor entities; astrocytomas and medulloblastomas. Furthermore, through our approach, the majority of currently proposed markers for ependymoma (e.g. nucleolin, nestin, Ki67 and laminin subunit A2) as well as all major key players of the phosphoinositide 3-kinase pathway (seemingly implicated in ependymoma), were definitely detected.

Pediatric brain tumors are a leading cause of cancer-related death in children (1). The application of high-throughput technologies (e.g. proteomics, and genomics) to the analysis of these tumor types has generated an abundance of molecular information, while in parallel, adequately continuing to provide knowledge on both the biological and clinical aspects of this devastating disease affecting children (2).  

Ependymoma, the third most common tumor in children, is thought to arise from ependymal cells in the wall of the cerebral ventricles or the spinal canal and therefore occurs most frequently in the posterior fossa or the spinal cord (3, 4). A variety of different sub-types of ependymomas have been identified, while the anaplastic variant seems to have the worse prognosis (5). Surgery remains the mainstay of treatment for ependymomas, while patients with posterior fossa ependymomas who have tumors amenable to gross total resection and are subsequently treated with radiotherapy, have a 70% or greater likelihood of long-term survival (6).
Due to the heterogeneity of the disease, its biological characteristics remain largely unknown and prognostic factors are basically based on clinical and histological criteria (i.e., age, extent of tumor resection, and histological grade). Therefore, biological, both genetic and proteomic, alterations that could be used to further characterize these tumors as well as identifying molecules that can be used as targets for therapy, need to be discovered.

Proteins, being the major conductors of genetic information and the molecules that can better reflect the functional status of the cell, are key targets in central nervous system (CNS) cancer research (7). This is why the elucidation of protein expression and their modifications is crucial in brain cancer biology, also aiding in discovery of predictors of cancer risk, detection of biomarkers for early diagnosis and identification of therapeutic targets (8). Proteomics, working together with genomics, may be able to redefine current ependymoma classifications and management protocols (9).

In our previous work, we reported on protein/proteomic signatures of pediatric astrocytomas and pediatric medulloblastomas, having had the opportunity to unravel parts of the molecular signature of these two distinct malignancy types, based on experiments utilizing two-dimensional gel-based protein separation and protein quantitation via image analysis and western blotting. It must, however, be underlined that the two-dimensional gel-based approach is biased to identify relatively high-abundant proteins and may miss on low-abundance regulatory molecules, including signal transducers, membrane proteins etc. Currently, gel-free approaches, namely liquid chromatography-tandem mass spectrometry (LC-MS/MS), are being widely used for clinical tissue and cell-line analyses (10).

The aim of the current study was two-fold: (i) to develop a sensitive and reproducible LC-MS/MS method for full in-depth proteomic screening of brain tumor tissues, and (ii) to build a high-confidence, in-depth proteome map from clinical cancer tissue specimens of ependymal origin, offering a first step towards their targeted protein validation in the clinical setting by the anticancer research community.

Materials and Methods

Tissue specimens. All samples were obtained from patients who underwent tumor resection surgery at Aghia Sophia Children’s Hospital, Athens, Greece. All procedures were in accordance with approved human subject guidelines, and were approved by the Ethical Committee of the Athenian University. After informed consent was obtained from the patients’ parents, samples were collected at initial diagnosis with no prior exposure to chemotherapy or radiation therapy.

All specimen analyzed in the study belonged to the cellular (WHO grade II) ependymoma subtype and all affected individuals were Caucasian (Table I). Resected tumors were stained with hematoxylin and eosin and examined by light microscopy.

Sample preparation. Prior to analyses, all tissue samples were washed in a sucrose buffer (HEPES, pH 7.5, 320 mM sucrose, 1 mM EDTA, 5 mM dithioerythritol, 1 mg/ml protease inhibitors) for removal of excess blood from tissue. Next, tissues were powderized through grinding in liquid nitrogen. Further homogenization for disruption of all remaining intact protein structures was performed by tip-sonication for three cycles, 18 seach, under 38% amplification. The lysis buffer used was 7 M urea, 1.5 M Tris-HCl, 0.1 M sodium dodecyl sulfate. The homogenate was left at room temperature for 1 h and centrifuged at 12,700 × g for 30 min. De-salting was performed with Ultrafree-4 centrifugal filter unit (Millipore, Billerica, MA, USA). The protein content of the supernatant was determined using the Bradford quantification method. Protein extraction was sequentially performed by addition of 150 μl of extraction buffer to the sample solution. Finally, 150 μg of protein was further processed for peptide generation.

Peptide generation. Proteins were reduced, in solution, with 0.1 mM DTE in Tris-HCl pH 6.8 for 30 min at 36°C. Proteins were then alkylated by the addition of 0.05 mM iodoacetamide for 30 min at room temperature in the dark. A trypsin solution (Roche, Hoffman-La-Roche, Basel, Switzerland) (final concentration 500 ng/μl) was added to the sample at a trypsin to protein ratio of 1:100, mixed by pipetting and digestion took place overnight at 36°C. The following day, trypsinization was terminated by the addition of 5% acetic acid (vol/vol). Finally, peptide-containing solutions were vacuum-dried for 1 h (until complete dryness). Powder was re-constituted in 100 μl of water with 0.1% formic acid. After cleaning for impurities by filtering through a Millex® syringe-driven filter unit, nanoLC-MS/MS analysis followed.

One-dimensional nano-liquid chromatography (1D-nanoLC) separation. Peptides generated in the previous step were separated in an Ultimate3000 system nanoLC system (Dionex; Thermo Scientific, Bremen, Germany). Peptides were loaded onto a C-18 pre-column (100 μm inner diameter ×2 cm; 100 Å, 3-μm-bead-packed, Acclaim PepMap 100; Thermo Scientific) at 10 μl/min in 99.9% water with 0.1% formic acid. After 6 min of desalting, the pre-column was switched online with the analytical C-18 column (75 μm ×50 cm; 100 Å, 2-μm-bead-packed Acclaim PepMap RSLC; Thermo Scientific) that was equilibrated with mobile phase A (99.9% water with 0.1% formic acid). Elution time for all runs was 360 min for under a non-linear gradient of mobile phase B (99.9% acetonitrile with 0.1% formic acid) (Table II) at a constant 300 nl/min flow rate.

Orbitrap mass spectrometry. Mass spectra of eluted peptides were collected in an Orbitrap Elite mass spectrometer (Thermo Scientific) fitted with a nano-spray source, practically as previously described by our group (11, 12). The instrument was run in a data-dependent acquisition mode with the XCalibur™ v.2.2 SP1.48 software (Thermo Scientific). Full-scan data were acquired on the 300-2,000 m/z range under 60,000 resolution and a maximum injection time of 100 ms. Data-dependent tandem MS for the 20 most intense ions was performed with higher-energy collision dissociation fragmentation in the Orbitrap at a resolving power of 15,000 and a collision energy of 36. Resulting fragments were analyzed on the Orbitrap; MS/MS spectra were acquired with 15,000 resolving power and a maximum injection time of 120 ms. Measurements were performed using m/z 445.120025 as lock mass.

128
functions and diseases that were most significant to our protein data sets. Proteins showing highest expression in ependymoma samples (false discovery rate of 30%) were considered for the analysis. The focus genes from all protein lists were overlaid onto a global molecular network, developed from information contained in the Ingenuity Pathway Knowledge Base and this generated networks based on the connectivity of the individual proteins according to their relevance with the tissues under study.

**Results**

In the present study, we embarked on analyzing the protein expression patterns of pediatric ependymoma tissues. Ten selected resected specimens were subjected to 1D-nanoLC-MS/MS analysis and to strengthen the quality and reproducibility of protein profiling of each sample/tissue, analysis consisted of two technical replicates. The methodology followed in order to increase the number of identifications, and in parallel save on analysis time and quantity of material required, consisted of experimenting with several sample preparation procedures with regard to the protein extraction process, as well as testing in LC parameters.

Initially, tissues were prepared under two different approaches, the first consisting of tissue homogenization in liquid nitrogen followed by protein extraction in 0.1 M Tris-HCl, supplemented with 4% SDS and 0.1 M DTE (buffer A) and the second consisting of homogenization in Wheaton grind homogenizer followed by extraction in 8 M urea in ammonium bicarbonate (50 mM) (buffer B) (Figure 1). Better results with regard to total protein identification numbers were achieved when the procedure including dissemination in liquid nitrogen and buffer A was followed.

Protocol optimization with regard to LC parameters included testing with a three-step non-linear gradient (Table II) against a classical linear 2-35% mobile phase B gradient. Results indicated that an even distribution across the chromatographic run was achieved in the first, tailored method, compared to MS1 ions detected using linear gradients. Therefore, chromatographic profile design of the
gradient offered a nearly constant number of unique peptides identified per minute of retention time. All optimized methods followed in the present study are described in detail in the Materials and Methods section.

After reaching a consensus with regard to optimizing the experimental procedure, all tissues were analyzed under the same protocol. The number of proteins identified in each tissue sample varied, with a mean of 4,000±150 protein groups and a mean of 25,000±550 peptides identified per sample. Only proteins (protein groups) identified/present in all samples were included in the final reference ependymoma protein database, consisting of 4,157 entries. An abstract of the final database showing protein coverage, unique peptides identified, peptide spectral matches, molecular weight and pI of identified proteins is given in Figure 2.

Furthermore, an investigation of the mRNA–miRNA interaction was carried out for each gene encoding the identified proteins. For this purpose, DIANA-TarBase v7.0, a database indexing more than half a million experimentally supported mRNA–miRNA interactions was used (13, 14). DIANA-TarBase v7.0 enables users to easily identify positive or negative experimental results, the utilized experimental methodology, experimental conditions including cell/tissue type and treatment. As a result, all experimentally validated miRNA:gene interactions for each human gene encoding the identified proteins is demonstrated in Table III.

Regarding GO terms, ependymoma proteins were grouped according to their biological process as well as localization. The majority of identified proteins (29%) are involved in cell metabolic processes, while a significant number (22.5%) are engaged in cell structure, 16.5% in localization procedures, 12% have biological regulatory roles, while others are connected to biogenesis (7%), development and stimulatory or immune responses (4%), or exert multiple functions (3%). Less frequently represented are proteins with biological adhesion (2%), proteins associated with apoptosis (2%) and cell reproduction (1%), as well as those associated with locomotion (1%) (Figure 3A).

In terms of their cellular distribution, a large proportion of proteins were found to be localized in organelle lumen (12.54%) and a greater number of proteins (14.8%) were components of the nucleolus. The majority of pediatric ependymoma proteins were found to be cytoplasmic (18.66%), while identified proteins were also present in other subcellular compartments such as the cytosol (13%), the mitochondrion (10%) and golgi apparatus (14%), and 7%
were membranous. Ten percent of identified proteins were extracellular, as well as comprising proteins of the endoplasmic reticulum (Figure 3B).

Ependymoma proteins were further clustered according to their biophysical characteristics; the assessed theoretical pI values predicted an even protein accumulation in both the acidic region (pI 4-7) and pI 8-10 region (Figure 3C).

The ependymoma protein database was further analyzed through the Ingenuity Pathway Knowledge Base. By this functional tool, protein biological functions and diseases that are most significant to the datasets are identified. The rank product list, representing proteins present in the ependymoma analyzed samples, generated the following top networks: (i) Cellular function and maintenance, post-translational modification, protein folding (Ingenuity Pathway analysis internal score, 60); (ii) dermatological diseases and conditions, inflammatory disease (score, 58); and (iii) cancer, hematological disease, reproductive system disease (score, 39). Cancer, dermatological diseases and gastrointestinal diseases are the main diseases connected to the list. The top canonical pathways associated with high significance are the 14-3-3-mediated signaling (p-value 3.25e-16) and the protein ubiquitination pathway (p-value 1.2e-11).

Several networks, implicating the analyzed molecules, showing protein–protein interactions, were generated by the software. A very interesting network implicating identified molecules significant in cell death of ependymal tissues is presented in Figure 4.

**Discussion**

In the present study, by employing a modified cutting-edge one-dimensional shotgun proteomic approach, we aimed to unveil for the first time the proteome of a pediatric brain tumor entity, generally unknown regarding its molecular-proteomic biological traits, pediatric ependymoma.
With regard to proteome optimization steps employed in the present study, aiming at reaching greater protein coverage without compromising on speed of analysis and sensitivity, we herein propose a universal sample preparation method for nanoLC-MS/MS analysis of pediatric brain tissues, which can, under certain conditions, be applied to all experiments on brain tissue specimens, whether malignant or not. Optimization steps were assessed on two parameters: (i) sample preparation by two different extraction buffers, and (ii) LC gradient conditions.

Under the concentrations provided, the proposed lysis buffer (buffer A) in combination with tissue severance in liquid nitrogen was optimal in attaining maximal dissociation of protein complexes, with adequate protein extraction, as well as solubility of the extracted material.

Regarding experimentation on LC parameters, it is well known that for complex peptide mixtures, conventional linear gradients produce an unequal spread of peptides, and peptide distributions deviate considerably from uniformity, with larger numbers of peptides eluting in the middle of the run, and relatively few peptides eluting in the beginning and towards the end of the gradient time. In the present study, we propose a three-step non-linear gradient that seems to provide an even distribution of all eluted ions across the chromatographic run compared to MS1 ions produced using linear gradients.

Under a constant flow rate of 300 nl/min, the improved chromatographic parameters were set to begin at 4% acetonitrile increasing linearly to 25% over 250 min, then to 55% over 80 min, and finally to 80% over 30 min. We also experimented on elution time duration and reached the conclusion that a clear and linear correlation between the chromatographic peak capacity in peptide elution (15) was achieved at 360 min of total elution time.

By following the above experimental protocol modifications regarding protein extraction and chromatographic parameters, we developed an LC-MS/MS method able to deliver more than 5,000 protein entries belonging to 4,155 protein groups and 25,345 peptides, thus mapping in-depth for the first time the greater part of the proteome of this pediatric-type human brain malignancy.

Previously, we had undertaken steps in studying the other two major pediatric brain tumor malignancies: pediatric astrocytoma (16) and pediatric medulloblastoma (15), by means of proteomics technologies. The current series of analyzed samples sheds light on similarities in protein patterns throughout the panel of the three pediatric brain tumor malignancies. It is disappointing that we cannot yet discuss in-depth differences of protein content (in a quantitative manner) between the three types, since the proteomic technologies employed for astrocytomas and medulloblastomas were limited.
medulloblastomas far lacked the forward/analytical character of the nanoLC-MS/MS procedure described herein.

Regarding the biological traits of the material under study, all major components of the mitogen-activated protein kinase pathway that had been reported to be de-regulated in grade 1 tumors (astrocytomas) (Q13418, Q04759, P12931, P63104, Q04917, P27348, P28482) by us and others (16-18), are present in the final list of ependymomas, further confirming the intermediate character of this type of malignancy (5).

Furthermore, regarding expression of heat-shock molecules, similarities were found in heat-shock protein 90 (P08238), 60-kDa heat-shock protein (P10809) and 70-kDa heat-shock protein (P34932), which were found to be equally expressed in low-grade gliomas (astrocytomas) and ependymomas, consistent with the general scheme that heat shock proteins are expressed as a response to various stress stimuli, including cancer. However, heat-shock protein 71-kDa (P11142), and heat-shock protein beta (P04792) and 90-alpha (P07900) were exclusively expressed in ependymomas.

Interestingly, both prohibitin (P35232), prohibitin 2 (Q99623), glial fibrillary acidic protein (GFAP; P14136) and vimentin (P08670) were among the proteins expressed in ependymomal tissues, similarly to astrocytomas. Prohibitin, a pleiotropic protein overexpressed in several tumor types, has been implicated in the regulation of cell proliferation, invasive migration and survival, and the ERK pathway (19).

Positivity of tumors for both immunohistochemical markers vimentin and GFAP revealed a similar character of ependymoma to that of astrocytoma, revealing a high requirement for chaperoning activity inside tumor cells, enhanced by the presence of heat-shock proteins (20, 21).

Expression of the galectin protein family is in tight correlation with human gliomas and relevant to the modulation of invasion of tumor astrocytes into brain parenchyma (22). Galectins are glycans-binding proteins highly expressed in several tumor types (including brain neoplasms) and have been correlated with adverse prognosis of certain tumor types; strong expression of galectin-3 has been found in astrocytomas and medulloblastomas (23). In our study, galectins 1, 3, 8, 9 and 9b were found to be present in our analyzed series of patients. Due to their unique structure, galectins can oligomerize forming lattices upon binding to multivalent oligosaccharides and influence several pathological events, such as tumorigenesis, invasion and metastasis (24).

Peroxiredoxins I-VI have recently been shown to have a role in the tumorigenesis of astrocytic brain tumors (25, 26). The presence of all peroxiredoxin molecules, except for peroxiredoxin-3, was noted in our studied ependymoma samples, in contrast to Haapasalo et al. who reported absence of peroxiredoxin 4 (26). Peroxiredoxin presence advocates the presence of defense in oxidative damage, to surpass the effect of oxidative stress taking place in ependymoma tissues.

A similar outline was depicted for similarities and differences regarding ependymal tissues and medulloblastoma. Coronin 1A, 1B, 1C and 2B were identified as components of ependymoma tissues. Coronin 1B (Q9BR76) is known to be implicated in contractility at adherens junctions and therefore influences apoptotic cell extrusion (27). Furthermore, coronin 1A (P31146), a newly identified p53 transcriptional target and a homotrimeric F-actin binding protein, important for cell migration and brain morphogenesis, whose expression has been implicated in diffuse glioma malignancies (28, 29) was found to be part of the ependymomal proteome; this specific molecule was also present in our previous analysis of medulloblastoma tissues (17).
Furthermore, drebrin (Q16643), an actin-binding protein involved in the regulation of actin filament organization, playing a significant role in cell motility and having been implicated in glioma cell invasiveness (28), has equally been found to be expressed in medulloblastoma and ependymoma tissues. Both drebrin and synaptophysin (P08247) are neuronal synaptic markers playing roles in microglial activation prior to any visible signs of neuronal cell death, including neuronal cleaved caspase-3 activation (31).

Similarly to medulloblastoma, programmed cell death 6-interacting protein (Q8WUM4), a key regulator of tumor growth and angiogenesis implicated in the PI3K pathway, protein lyric (Q86UE4), a protein that activates the nuclear factor-κB transcription factor and promotes anchorage-independent growth of immortalized melanocytes and astrocytes and secreted protein acidic and rich in cysteine (SPARC) (P09486), an inducer of neuronal differentiation as a cell defense mechanism against tumor transformation, were all found to be present in ependymoma tissues (32-34).

Overall, all putative biological markers proposed through genomic studies/approaches identified in larger cohorts of samples including nucleolin (P19338) (35), Ki-67 (P46013) (36), nestin (P48681) (37) and laminin subunit A2 (P24043) (38) were identified as being present in the our analyzed
samples, thus further confirming the analytical power of the method of analysis proposed herein.

The PI3K pathway is one of the most commonly activated pathways in cancer, including glioma and medulloblastoma cancer types (17, 39, 40). PI3Ks are lipid kinases that transduce signals from growth factors and cytokines influencing diverse biological functions, including cellular proliferation, survival, and motility. The PI3K pathway has been reported to be activated in a large number of ependymoma cases examined by polymerase chain reaction and immunohistochemical analyses (41). It is of great importance that the nanoLC-MS/MS method developed in the present study allows for definite identification of key molecules of the specific pathway, as well as being present in the final protein list of ependymomas, reported in our present study.

Whether future molecular pathophysiology experiments lead research of this specific brain malignancy towards the PI3K pathway as a potential therapeutic target and potential prognostic marker for ependymoma, or towards any other relevant pathway or isolated molecule or panel of molecules, one fact is certain: proteomics, and more specifically, high-resolution, unbiased, accurate and multifaceted analysis. Cancer Genomics Proteomics 12(6): 369-384, 2015.

Acknowledgements
The Authors would like to thank all patients and their families without whom advance in pediatric brain tumor research would not be possible.

References
1 Firme MR and Marra MA: The molecular landscape of pediatric brain tumors in the next-generation sequencing era. Curr Neurol Neurosci Rep 14(9): 474, 2014.
2 Anagnostopoulos AK and Tsangaris GT: Proteomics of pediatric brain tumors. Exp Reviews Proteomics 11(5): 641-648, 2014.
3 de Bont JM, den Boer ML, Kros JM, Passier MM, Reddingius RE, Smitt PA, Luider TM and Pieters R: Identification of novel biomarkers in pediatric primitive neuroectodermal tumors and ependymomas by proteome-wide analysis. J Neuropathol Exp Neurol 66(6): 505-516, 2007.
4 Merchant TE and Fouladi M: Ependymoma: New therapeutic approaches including radiation and chemotherapy. J Neurooncol 75: 287Y99, 2005.
5 de Bont JM, Packer RJ, Michiels EM, den Boer ML and Pieters R: Biological background of pediatric medulloblastoma and ependymoma: a review from a translational research perspective. Neuro Oncol 10(6): 1040-1060, 2008.
6 Nieder C, Andratschke NH and Grosu AL: Re-irradiation for recurrent primary brain tumors. Anticancer Res 36(10): 4985-4995, 2016.
7 Da Costa GG, Gomich TG, Kavinski R, Santos Sousa K, Kukolj C, De Lima RS, De Andrade Urban C, Cavalli IJ and Ribeiro EM: Comparative proteomics of tumor and paired normal breast tissue highlights potential biomarkers in breast cancer. Cancer Genomics Proteomics 12(5): 251-261, 2015.
8 Weidle UH, Birzle F, Kollmorgen G and Rüger R: Dissection of the process of brain metastasis reveals targets and mechanisms for molecular-based intervention. Cancer Genomics Proteomics 13(4): 245-258, 2016.
9 Anagnostopoulos AK, Vougas K, Kolialexi A, Mavrou A, Foundoulakis M and Tsangaris GT: The protein profile of the human immature T-cell line CCRF-CEM: Cancer Genomics Proteomics 2(4): 271-300, 2005.
10 Polisetti RV, Gautam P, Sharma R, Harsha HC, Nair SC, Gupta MK, Uppin MS, Challa S, Puligopu AK, Ankathi P, Purohit AK, Chandak GR, Pandey A and Sirdeshmukh R: LC-MS/MS analysis of differentially expressed glioblastoma membrane proteome reveals altered calcium signaling and other protein groups of regulatory functions. Mol Cell Proteomics 11(6): M111.03565, 2012.
11 Anagnostopoulos AK, Stravopodis DJ and Tsangaris GT: Yield of 6,000 proteins by 1D nLC-MS/MS without pre-fractionation. J Chromatogr B Anal Technol Biomed LifeSci. pii: S1570-0232(16)30656-0. 2016.
12 Velentzas AD, Anagnostopoulos AK, Velentzas PD, Mepouk VE, Sagioglou NE, Tsioka MM, Katarachia S, Manta AK, Konstantakou EG, Papassideri IS, Tsangaris GT and Stravopodis DJ: Global proteomic profiling of Drosophila ovary: a high-resolution, unbiased, accurate and multifaceted analysis. Cancer Genomics Proteomics 12(6): 369-384, 2015.
13 Paraskevopoulou MD, Vlachos IS and Hatzigeorgiou AG: DIANA-TarBase and DIANA Suite Tools: Studying experimentally supported microRNA targets. Curr Protoc Bioinformatics 55: 12.14.1-12.14.18, 2016.
14 Vlachos IS, Paraskevopoulou MD, Karakouki D, Georghiakas G, Vergoulis T, Karoulis K, Anastasopoulos IL, Maniou S, Karathanou K, Kalfakakou D, Fygas A, Dalamagas T and Hatzigeorgiou AG: DIANA-TarBase v7.0: indexing more than half a million experimentally supported miRNA:mRNA interactions. Nucleic Acids Res 43(Database issue): p. D153-159. 2015.
15 Thakur SS, Geiger T, Chatterjee P, Bandilla P, Fröhlich F, Cox J and Mann M: Deep and highly sensitive proteome coverage by LC-MS/MS without prefractionation. Mol Cell Proteomics 10(8): M110.003699, 2011.
16 Anagnostopoulos AK, Dimas KS, Papathanassiou C, Braoudaki M, Anastasiadou E, Vougas K, Karamolegou K, Kontos H, Prodromou N, Tzortzatou-Stathopoulou F and Tsangaris GT: Proteomics studies of childhood pilocytic astrocytoma. J Proteome Res 10(5): 2555-2565, 2011.
17 Anagnostopoulos AK, Papathanassiou C, Karamolegou K, Anastasiadou E, Dimas KS, Kontos H, Koutsopoulos A, Prodromou N, Tzortzatou-Stathopoulou F and Tsangaris GT: Proteomic studies of pediatric medulloblastoma tumors with 17p deletion. J Proteome Res 14(2): 1076-1088, 2015.
18 Pfister S, Janzarik WG, Remke M, Ernst A, Werft W, Becker N, Toedt G, Wittmann A, Krazt C, Olbrich H, Ahmadi R, Thieme B, Joos S, Radl wimmer B, Kulozik A, Pietsch T, Herold-Mende.
C. Gnekow A, Reifenberger G, Korshunov A, Scheurlen W, Omran H and Lichter P: BRAF gene duplication constitutes a mechanism of MAPK pathway activation in low-grade astrocytomas. J Clin Invest 118(5): 1739-1749, 2008.

19 Cao Y, Liang H, Zhang F, Luan Z, Zhao S, Wang XA, Liu S, Bao R, Shu Y, Ma Q, Zhu J and Liu Y: Prohibitin overexpression predicts poor prognosis and promotes cell proliferation and invasion through ERK pathway activation in gallbladder cancer. J Exp Clin Cancer Res 35: 68, 2016.

20 Fernando G, Paul F, Laura J, Alejandra AM, Gabriela M and Alberto PL: Is the WNT/β catenin signalling pathway active in seminoma? An immunohistochemical study. J Cancer Res Ther 12(2): 1075-1079, 2016.

21 Palm T, Figarella-Branger D, Chapron F, Lacroix C, Gray F, Scaravilli F, Ellison DW, Salmon I, Vikkula M and Godfraind C: Expression profiling of ependymomas unravels localization and tumor grade-specific tumorigenesis. Cancer 115(17): 3955-3968, 2009.

22 Ronive S, Belot N, Decaestecker C, Lefranc S, Gourdow L, Micik S, Maugue CA, Kaltner H, Ruchoux MM, Danguy A, Gabius HJ, Salmon I, Kiss R and Camby I: Galectin-1 is highly expressed in human gliomas with relevance for modulation of invasion of tumor astrocytes into the brain parenchyma. Glia 3: 241-255, 2001.

23 Borges CB, Bernardes ES, Latorraca EF, Becker AP, Neder L, Chammans R, Roque-Barreira MC, Machado HR and de Oliveira RS: Galectin-3 expression: a useful tool in the differential diagnosis of posterior fossa tumors in children. Childs Nerv Syst 27(2): 253-257, 2011.

24 Pereira JX, Azeredo MC, Martins FS, Chammans R, Oliveira FL, Santos SN, Bernardes ES, El-Cheikh MC: The deficiency of galectin-3 in stromal cells leads to enhanced tumor growth and bone marrow metastasis. BMC Cancer 16: 636, 2016.

25 Jarvela S, Rantala I, Rodriguez A, Kallio H, Parkkila S, Kinnula VL, Soini Y and Haapasalo H: Specific expression profile and prognostic significance of peroxiredoxins in grade II-IV astrocytic brain tumors. BMC Cancer 22: 100-104, 2010.

26 Haapasalo T, Nordfors K, Järvelä S, Kok E, Sallinen P, Kinnula J, Rantala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rodriguez A, Kallio H, Parkkila S, Kinnula S, Rautala I, Rod...