Proteomic analysis of glioblastomas: what is the best brain control sample?

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Abstract

Glioblastoma (GB) is the most frequent and aggressive tumor of the central nervous system. There is currently growing interest in proteomic studies of GB, particularly with the aim of identifying new prognostic or therapeutic response markers. However, comparisons between different proteomic analyzes of GB have revealed few common differentiated proteins. The types of control samples used to identify such proteins may in part explain the different results obtained.

We therefore tried to determine which control samples would be most suitable for GB proteomic studies. We used an isotope-coded protein labeling (ICPL) method followed by mass spectrometry to reveal and compare the protein patterns of two commonly used types of control sample: GB peritumoral brain zone samples (PBZ) from six patients and epilepsy surgery brain samples (EB) pooled from three patients. The data obtained were processed using AMEN software for network analysis.

We identified 197 non-redundant proteins and 35 of them were differentially expressed. Among these 35 differentially expressed proteins, six were over-expressed in PBZ and 29 in EB, showing different proteomic patterns between the two samples. Surprisingly, EB appeared to display a tumoral-like expression pattern in comparison to PBZ.

In our opinion, PBZ may be more appropriate control sample for GB proteomic analysis.

Significance

This manuscript describes an original study in which we used an isotope-coded protein labeling method followed by mass spectrometry to identify and compare the protein patterns in two types of sample commonly used as control for glioblastoma (GB) proteomic analysis: peritumoral brain zone and brain samples obtained during surgery for epilepsy. The choice of control samples is critical for identifying new pronostic and/or diagnostic markers in GB.

Keywords:

Proteomic, glioblastoma, ICPL, epilepsy

Introduction

Glioblastoma (GB) is the most frequent and aggressive tumor of the central nervous system. Despite the development of new therapies, the prognosis remains poor, with a mean progression-free survival of 7 months and an average survival of 12 to 15 months [1, 2]. Even following gross total resection and optimal adjuvant treatment, recurrence is extremely common, mainly from the margin of the resection cavity [3-5].

GB is a very hetererogenous groups of tumors [6], involving different zones; both genomic [7, 8] and proteomic [9-11] approaches have been used to study these tumors. These analyses led to the identification of different markers, allowing the characterization of different subtypes of GBs and tumoral mechanisms, and may serve as a basis for the development of new therapies focused on the molecular, genetic and proteomic particularities of GB.

In one of our previous proteomic studies, we used an isotope-coded protein label (ICPL) method to compare three areas of GB: the tumor zone (TZ), the interface zone between the tumor and the parenchyma (IZ) and the peritumoral brain zone (PBZ). We successfully identified 35 proteins over-expressed in the core of the tumor by comparison with the periphery and showed that 23 of these belong to a cohesive network of physically interacting proteins linked to several cellular functions [10].

However, few of the 35 proteins that we found to be altered in TZ are the same as those identified by previous studies (Table 1). For example, Khalil [12] used 2DE with MALDI-TOF MS and LC-MS/MS to analyze 30 GB samples with seven control samples obtained from epilepsy surgery for reference. Forty-six differentially expressed proteins were identified of which only ten proteins were in common with our study (β-actin, CKB, GDI1, ALDOA, 14-3-3γ, ATP5A1, ALB, GFAP, NEFL, ENO1). Except for β-actin, most of these proteins showed a different pattern of expression to that described in this previous study. Indeed, we found these proteins to be over-expressed in TZ versus PBZ whereas Khalil [12] observed under-expression in GB samples versus control epilepsy samples. To understand the apparent differences in the protein expression patterns between the two

studies, we conducted a bibliographical search for proteomic analyses of fresh brain tumor samples (reported in Table 1). This analysis revealed substantial heterogeneity in results associated with the different proteomic analysis techniques employed and the control tissues used. Indeed, differences in both the analytical methodologies and the control tissues used may explain the only weak similarities between proteomic patterns reported by the various studies.

Obviously, normal live brain samples are not available to be used as control samples under all circumstancies, and consequently the control samples commonly used in GB proteomic studies include brain tissue obtained during surgery for epilepsy (EB) or from the walls of the resection cavity during GB surgery (PBZ), with the informed consent of the patient.

However, it is unclear whether PBZ or EB brain samples, commonly used as controls, can be considered to be "normal" brain tissue, and therefore whether they are appropriate for proteomic comparisons and describing the differential proteomic expression pattern of brain tumors.

The aim of this study was to analyze and compare the protein expression patterns of these two control tissues (PBZ versus EB) using the ICPL proteomic method, and to determine which is the most suitable for use as control tissue for proteomic analyses of brain tumors.

Materials and Methods

Clinical materials

Six patients whose diagnosis of primary GB (WHO 2007 classification) was confirmed by a central committee of neuropathologists and three patients undergoing epilepsy surgery were included in the study. This study was approved by the relevant ethics committee (CPP Ouest II, Angers, France) and all patients signed an informed consent form for participation in this study.

The tumoral zone and PBZ from GB were defined on preoperative T1 gadolinium-enhanced 3D MRI. Stereotaxic biopsies were performed in the operating theater, by computer-assisted neurosurgery (BrainLab®, La Défense, France). EB was obtained from cortical resection during

Reference	Year	n	WHO grade	Method	Control	Proteins Differentially expressed	Proteins in common*
[27]	2011	5	GB	2DGE, MALDI-TOF MS, 2D-PAGE, Western Blot	EB	22	4
[28]	2009	27	GB	Western Blot	Cancer genome atlas	55	1
[29]	2009	1	GB	HPLC-ESI-MS/MS	PBZ	15	-
[30]	2009	3	GB	2DGE, MALDI-TOF MS	PBZ	8	1
[12]	2007	41	24 primary GBs 4 secondary GBs 4 grade III 2 grade II 7 epilepsy	2D-PAGE, 2DGE, MALDI-TOF MS	EB	91	9
[31]	2007	20	10 GBs 10 controls	Nano-LC prior to MALDI-TOF/TOF	Samples from different patients with a variety of CNS conditions	16	0
[32]	2005	20	10 grade IV 10 grade II	2DGE, LC–ESI–MS/MS, Western Blot	PBZ	15	2
[33]	2005	13	GBs	2DGE	PBZ	19	1
[34]	2005	127	57 GBs 22 grade III glioma 29 grade II glioma	MALDI-TOF	19 patients undergoing surgery for "non-neoplastic diseases"	24	0
[35]	2005	27	10 GBs 14 grade III 1 grade II 2 grade I	2DGE, MALDI-TOF MS	EB	29	4
[36]	2004	85	52 GBs 13 grade III 10 grade II 10 epilepsy	2DGE, MALDI-TOF MS	PBZ	37	2
[37]	2004	18	4 GBs 2 oligo II 2 AO grade III 2 embryonal carcinoma 1 pheochromocytoma 1 DNET 1 gemistocytic astrocytoma grade II	MALDI-MS	EB	Identification of protein patterns without protein characterization	-
[38]	2003	5	2 GBs 2 grade III 1 grade I	2DGE, MALDI-TOF MS	PBZ from the same patient	15	1
[39]	2003	4	4 GBs	SELDI-TOF-MS	None	Identification of protein profiles without protein characterization	-
[40]	2001	94	56 GBs 13 AAIII 25 low-grade gliomas	Western Blot analysis	Lysates from 16 week old fetuses	14	0

Table 1: Overview of the GB biopsy proteomic literature and comparison of the proteins identified with our previous study* [10]

GB: glioblastoma; AA: anaplastic astrocytoma; AO: anaplastic oligodendroglioma; oligo: oligodendroglioma; DNET: Dysembryoplastic neuroepithelial tumor; 2DGE: 2D Gel electrophoresis; ICPL: isotope coded protein labelling; LC-ESI: liquid chromatography electrospray ionization; MALDI-TOF: matrix-assisted laser desorption ionization time of flight; nano-LC: nanoliquid chromatography; SELDI-TOF: surface enhanced laser desorption ionization time of flight; PBZ: peritumoral brain zone, EB: peripheral epilepsy surgery brain zone.

surgery for epilepsy after identification of the epileptic cradle using per-operative electroencephalograms and electrostimulation.

Histological analysis and protein extraction were performed for each biopsy specimen. For histological analysis, formalin-fixed, paraffin-embedded sections of the biopsy specimens were stained with hematoxylin-phloxin-saffron.

Tissue protein extraction

Protein extracts of tissue samples were prepared as previously described [10]. Briefly, cell pellets from PBZ and EB samples were resuspended in cold lysis buffer (6 M guanidine HCl, pH 8.5, cells/buffer: 1/2.5(v/v)) and sonicated on dry ice with an ultrasonic processor (Bioblock Scientific, Illkirch, France) six times for 10 sec with 30 sec pauses between using a microtip setting power level at 40% pulse duration. The homogenates were centrifuged (15,000 g, 30 min, 4°C) and the resulting supernatants were then ultracentrifuged (105,000 g, 1 h, 4°C). Protein concentrations in the resulting supernatants were measured with a BioRad Protein Assay Kit (BioRad, Marnes-la-Coquette, France) according to the manufacturer's instructions. The samples from the three patients undergoing epilepsy surgery were pooled.

ICPL labeling and protein digestion

The experimental design and the ICPL method are described in Table 2. ICPL labeling was performed on 50 µg of PBZ or pooled EB samples as previously described (Com et al., 2012), according to the experimental design described in Table 2. Labeled proteins (50 µg) were separated by SDS-PAGE in 12% precast gels (GeBeGel, Gene Bio Application), which was then stained with Coomasie blue R-350 using the EZBlue gel staining reagent (Sigma-Aldrich, Saint Quentin Fallavier, France). Entire gel lanes were cut into 20 sections, which were washed in different ACN/100 mM NH_4HCO_3 solutions. In-gel digestion was performed overnight at 37°C with modified trypsin (Promega, Charbonnières-lès-Bains, France) following a previously described protocol (13). Proteolytic peptides were then extracted from the gel sections by sequential incubation in the following solutions: ACN/H_20/TFA, 70:30:0,1 (v/v/v), 100% ACN and

ACN/H_20/TFA, 70:30:0,1 (v/v/v), and the extracts were concentrated by evaporation down to a final volume of 30 µL.

	L	M	H
Reaction 1	EB pool	GB3-PBZ	GB16-PBZ
Reaction 2	GB10-PBZ	EB pool	GB25-PBZ
Reaction 3	GB22-PBZ	GB26-PBZ	EB pool

Table 2: Experimental design of the ICPL labelling for each sample
L: light ICPL reagent, M: medium ICPL reagent, H: heavy ICPL reagent, GB-PBZ: glioblastoma-peritumoral brain zone, EB pool: peripheral epilepsy surgery brain samples pooled from three patients.

GLC-MS/MS analysis and protein identification and relative quantification

Proteolytic mixtures were analyzed on a nano-HPLC system (Ultimate 3000, Dionex, Jouy-en-Josas, France) coupled on-line with an Esquire HCT Ultra PTM Discovery mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany), equipped with a nanoflow ESI source and an ion trap analyzer (ITMS) as previously described [10]. The EsquireControl™ software (Bruker Daltonik GmbH) automatically alterned MS and MS-MS acquisitions and was tuned to preferentially subject ICPL-labeled peptides to MS-MS acquisitions. DataAnalysis™ 3.4 software (Bruker Daltonik GmbH) was used to create the peak lists from raw data. For each acquisition, a maximum of 700 compounds was detected with an intensity threshold of 100,000 and the charge state of precursor ions was automatically determined by resolved-isotope deconvolution. ProteinScape™ 2.0 software (Bruker Daltonik GmbH) was used to submit MS/MS data to the Swiss-Prot database (version 70, November 2011, Homo Sapiens taxonomy, 20257 sequence entries) and the randomized version of this database (decoy) to determine the false positive rate (FPR), defined as the number of validated decoy hits / (number of validated targets hits + number of decoy hits) * 100, using the Mascot algorithm (Mascot server v2.2, http://www.matrixscience.com) as previously described (Com et al., 2012). Given that modification of lysine residues by ICPL labeling prevents their cleavage by trypsin, arginine C was selected as the enzyme with one allowed miscleavage. Carbamidomethylation of cysteines was set as a fixed modification, and labeling of lysine residues

by light (L), medium (M) or heavy (H) ICPL reagents, and methionine oxidation were considered as variable modifications. The mass tolerance for parent and fragment ions was set to 0.25 and 0.5 Da, respectively. Peptide identifications were accepted if the individual ion Mascot scores were above 30 or above the identity threshold (the ion score is -10*log (p), where p is the probability that the observed match is a random event, p-value <0.05). The ProteinExtractor algorithm (14) was used to compile identified peptides to proteins as previously described [10]. Every protein reported was identified by at least one peptide with significant ion Mascot score (above the identity threshold) and which cannot be mapped to a higher-ranking protein already in the result list, and protein identifications were accepted if the FPR of the search was lower than 1%.

WarpLC 1.2 software (Bruker Daltonik GmbH) was used for relative protein quantification; this software automatically calculates H/L, M/L and H/M ratios by comparing the relative intensities of m/z ratios corresponding to the labeled peptides observed on MS spectra using DataAnalysis 3.4 software using previously described parameters [10]. The minimum differences in H/L, M/L, and H/M ratios associated with significant differences in protein expression were determined by calculating the technical variability of our system as previously described [10] and the significant threshold was fixed at 2 standard deviations from the normalized median of each H/L, M/L and H/M ratio; this gavethresholds od 1.41 for over-expressed proteins and 0.71 for under-expressed proteins (data not shown).

Gene ontology term enrichment and network analysis

The Annotation, Mapping, Expression end Network (AMEN) suite of software tools (16) was used to assess biological process GO term enrichment in the GB-PBZ / EB differential protein group using the International Protein Index human proteome for reference (release 3.8) (20). To be significantly over-represented in a group of genes, a GO term should have a p-value (adjusted with FDR by the Benjamin-Hochberg Method) lower than 0.01 and at least three proteins had to be associated with the annotation involved. A high Ontology Specific Information Rate (OSIR) cut-off, ≥ 0.4, was selected to avoid redundancy between closely related terms.

The network representation was drawn using AMEN and seven protein interaction databases: IntAct (release 2010-03, http://www.ebi.ac.uk/intact), MINT (release 2010-03, http://160.80.34.4/mint/Welcome.do), BioGRID (release 2010-03, http://thebiogrid.org), DIP (release 2010-04, http://dip.doe-mbi.ucla.edu), HPRD (release 2010-04, http://www.hprd.org), CORUM (release 2010-04, http://mips.helmholtz-muenchen.de/genre/proj/corum), and MatrixDB (release 2010-04, http://matrixdb.ibcp.fr).

Results and discussion

This study is, to our knowledge, the first to compare the proteomic profiles of peritumoral brain zone tissue from GB and brain tissue samples obtained after epilepsy surgery to assess which is the most suitable for use as control samples for proteomic studies of GB.

Macroscopically, PBZ samples are composed of a mixture of grey and white matters but the proportion of white matter is greater because of the usual localization of GB in the brain.. As GB is an highly infiltrative tumor, histological analysis of PBZ can show microsatelite tumors or isolated infiltrated GB cells. For all the samples included in this study, histological analysis indicated an infiltration of tumor cells in two PBZ samples only (between 5-10%) (data not shown). However, this infiltration was too low to alter the genomic and proteomic profiles of PBZ. In fact, while array CGH analysis of TZ indicated gain of chromosome 7 and loss of chromosome 9 and 10, no such genomic aberrations were observed in corresponding PBZ (data not shown). As epileptic zone are usually cortical, EB samples are principally composed of grey matter with a small contingent of white matter. Consequently, these two types of brain tissue sample are divergent, with different cytoarchitectural organizations and containing different types of neural cells.

We used the ICPL technique to compare the proteomes of these two types of brain tissue sample because it allows high-throughput, quantitative proteome profiling in an acurate and reproducible manner for up to four different samples [13]. Unlike other MS-based differential proteomic approaches, this technique can be applied both to cell cultures and to tissue samples and provides

information about protein isoforms [14, 15]. The ICPL technique is is also informative about non-differential proteins thanks to the simultaneous quantification and identification.

By querying the human Swiss-Prot database, we identified 197 non-redundant proteins with a score above the identity threshold and with a FDR < 1% (supplementary table 1). The numbers of identified and quantified proteins per patient are reported in Table 3.

			PBZ/EB		
	# identified proteins[b]	# quantified proteins[c]	< 0.71[d]	>0.71 and <1.41[e]	>1.41[f]
GB3[a]	145	72	42	19	11
GB10[a]	123	58	36	10	12
GB16[a]	145	72	22	40	10
GB22[a]	116	55	36	11	8
GB25[a]	123	58	34	12	12
GB26[a]	116	55	46	6	3

Table 3: Number of identified and quantified proteins for each patient

[a] The anonymous designation of each patient is reported in the first column, [b] Number of unique identified proteins, [c] Number of unique quantified proteins, [d] Number of unique quantified proteins with a ratio < 0.71 (expression decrease above 41%), [e] Number of unique quantified proteins with a ratio > 0.71 and <1.41 (non modulated expression), [f] Number of unique quantified proteins with a ratio > 1.41 (expression increase above 41%)

To assess the differential expression of proteins between PBZ and EB samples, we selected proteins with an average ratio, in six analyzed patients, of > 1.41 or < 0.71, with individual ratios > 1.41 or < 0.71 in at least 3/6 patients with a minimum of two peptides in at least 50% of the patients. On this basis, we identified 35 proteins the expression of which differed between PBZ and EB. Most of these proteins (29/35 proteins, or 83%) were more weakly expressed in PBZ than EB, and only six were more strongly expressed (Table 4).

AMEN software was used to evaluate GO term enrichment. The over-represented biological functions (p-value < 0.01) in EB are associated with energy metabolism, nervous sytem development, synaptic transmission, cellular transport and protein folding and processing (Figure 1). We previously observed these biological functions as being up-regulated in GB biopsies by

comparison with PBZ samples used for reference [10]. Furthermore, we identified a functional network including 21 of the 35 proteins differentially expressed between PBZ and EB (Figure 2).

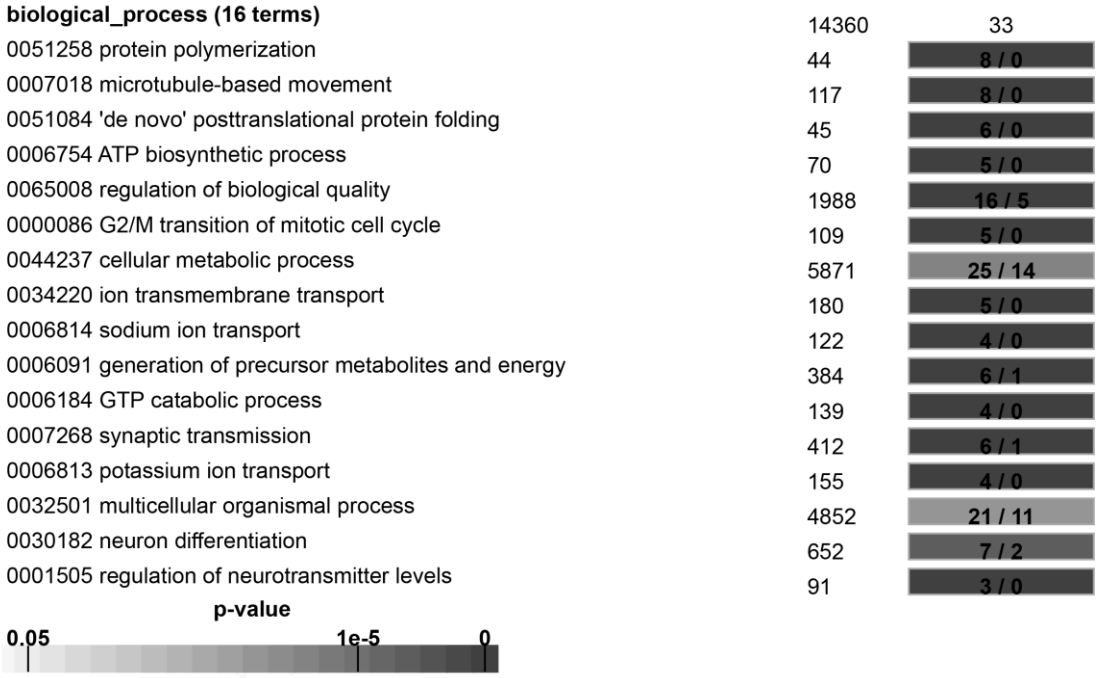

Figure 1: Biological processes involving the differentially expressed proteins

The color scale represents the down (in blue) or up-regulation (in red) with their respective corrected p-value for each GO term. Bold characters display a significant enrichment.

An analysis of the literature revealed that some of the six proteins up-regulated in GB-PBZ are ubiquitous, components of basic cellular pathways like DNA folding for histones (HIST1H2AC and HIST1H4A) or involved in the regulation of the osmotic pressure of blood, such as albumin. We will not consider these proteins further. The up-regulation of the myelin basic protein (MBP) and the glial fibrillary acidic protein (GFAP) observed in PBZ may be related to the fact that this zone contain more white matter than EB. . Note, however, that two of the up-regulated proteins, the crystallin B α-chain (CRYAB) and the histone H3F3A have known oncogenic roles.

The histone H3F3A was not considered to be an oncogene until recently when two publications reported that it is a specific and reproducible marker of pontine GB [16, 17]. These articles document two mutations, K27M and G64V, in a large cohort of children with pediatric GB. They also report that the K27M mutation is preferentially associated with cases with older onset, during

teenage, and a hemispheric localization [16]. Therefore, the histone H3F3A up-regulation in GB-PBZ/EB we observed is of interest. Possibly, this up-regulation could be the result of one of these mutations. Unfortunately, the tryptic peptides which would be mutated were not detected in our experiments.

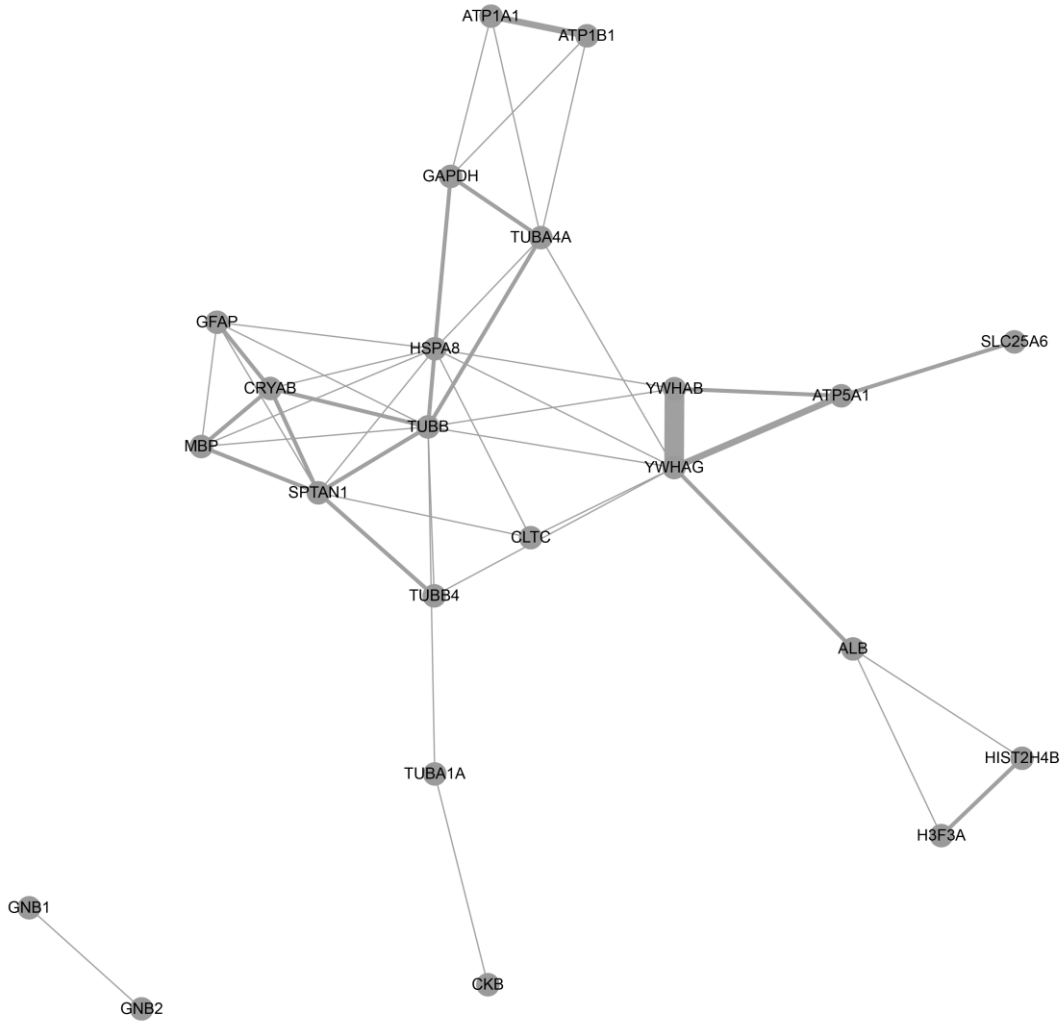

Figure 2: Interaction network representation of the differentially expressed proteins using the AMEN software

CRYAB is the major protein of the eye lens and also a chaperone belonging to the small heat shock protein family. In the CNS, CRYAB has a protective role in autoimmune demyelination [18]. CRYAB has been found in various types of solid tumor as a novel protein and may serve as a prognostic marker [19, 20]. CRYAB is also modulated by hypoxia [21, 22] and regulates tumor angiogenesis [23].

Our analysis of the literature for the 29 proteins up-regulted in EB versus PBZ indicated that only a few have a role in the pathogenesis of some forms of epilepsy: solute carrier family 25 member 6 (SCL25A6), Ras-related C3 botulinum toxin substrate 3 (Rac3), TUBA1A, spectrin alpha chain (SPTAN1) and GAPDH.

Surprisingly, we observed that several proteins over-expressed in EB have been implicated in the oncogenesis and in the pathophysological mechanisms of brain tumors; these proteins included 14-3-3 proteins β and γ, the ATPase Na+/K+ transporting alpha-1 and alpha-2 polypeptides (ATP1A1 and ATP1A2), GAPDH, the heat shock 70 kDa protein 8 (HSPA8), phosphatidylethanolamine binding protein 1 (PEBP1/RKIP) and the tubulin subtypes identified (TUBA1A, TUBA1B, TUBA4A, TUBAL3, TUBB, TUBB2A, TUBB3 and TUBB4).

These various findings demonstrate that the proteomes of PBZ and EB differ, but that EB has a more "tumoral" protein expression pattern, whereas PBZ seemed to have a more conventional proteomic profile. The up-regulation of oncogenic proteins such as Fos and Jun has already been observed in the dendate gyrus of animal models of epilepsy [24, 25]. Therefore, in our opinion, PBZ appears to be a more suitable control tissue than EB for proteomic studies of GB. However, considering PBZ as a "normal" tissue is likely to be erroneous. GB generally recurs at the resection margin, strongly suggesting that the PBZ is not "normal" [3-5]. Furthermore, we have isolated a new cell population from PBZ; these cells, named GB-associated stromal cells (GASCs), have properties similar to those of cancer-associated fibroblasts (CAFs). Like CAFs, GASCs express mesenchymal markers and have tumor-promoting effects [26].

Post-mortem brain tissue obtained from autopsy is a potential alternative source of brain control samples. However, Skold and collaborators [27] showed that several highly abundant proteins are enzymatically degraded in the brain within minute of death, such that the proteome rapidly differs from that normally present *in vivo*.

		PBZ/EB				
Accession[a]	Protein Name[b]	# ratio[c]	geometric mean[d]	CV[e]	ratio > 1.41 or < 0,71 / # patient[f]	% ratio quantified with 1 peptide[g]
Up-regulated proteins						
CRYAB_HUMAN	Alpha-crystallin B chain OS=Homo sapiens GN=CRYAB PE=1 SV=2	4	2,45	0,34	4/6	50%
H2A1C_HUMAN	Histone H2A type 1-C OS=Homo sapiens GN=HIST1H2AC PE=1 SV=3	4	2,43	0,35	3/6	50%
H33_HUMAN	Histone H3.3 OS=Homo sapiens GN=H3F3A PE=1 SV=2	6	2,13	1,13	4/6	0%
H4_HUMAN	Histone H4 OS=Homo sapiens GN=HIST1H4A PE=1 SV=2	6	2,05	0,81	4/6	0%
ALBU_HUMAN	Serum albumin precursor - Homo sapiens (Human)	6	2,04	1,01	4/6	0%
GFAP_HUMAN	Glial fibrillary acidic protein OS=Homo sapiens GN=GFAP PE=1 SV=1	6	1,70	0,87	4/6	0%
MBP_HUMAN	Myelin basic protein OS=Homo sapiens GN=MBP PE=1 SV=3	6	1,27	0,61	3/6	0%
Down-regulated proteins						
PRDX2_HUMAN	Peroxiredoxin-2 OS=Homo sapiens GN=PRDX2 PE=1 SV=5	6	0,81	0,56	3/6	0%
ENOA_HUMAN	Alpha-enolase - Homo sapiens (Human)	6	0,71	0,41	3/6	0%
CLH1_HUMAN	Clathrin heavy chain 1 OS=Homo sapiens GN=CLTC PE=1 SV=5	6	0,66	0,26	3/6	33%
DYN1_HUMAN	Dynamin-1 OS=Homo sapiens GN=DNM1 PE=1 SV=2	4	0,66	0,43	3/6	0%
1433B_HUMAN	14-3-3 protein beta/alpha OS=Homo sapiens GN=YWHAB PE=1 SV=3	4	0,58	0,50	3/6	0%
1433G_HUMAN	14-3-3 protein gamma OS=Homo sapiens GN=YWHAG PE=1 SV=2	6	0,58	0,37	4/6	0%
TBA4A_HUMAN	Tubulin alpha-4A chain OS=Homo sapiens GN=TUBA4A PE=1 SV=1	6	0,57	0,57	4/6	0%
TBB3_HUMAN	Tubulin beta-3 chain OS=Homo sapiens GN=TUBB3 PE=1 SV=2	6	0,56	0,40	5/6	0%
PEBP1_HUMAN	Phosphatidylethanolamine-binding protein 1 - Homo sapiens (Human)	4	0,56	0,24	3/6	50%
TBB4_HUMAN	Tubulin beta-4 chain OS=Homo sapiens GN=TUBB4 PE=1 SV=2	6	0,56	0,36	5/6	0%
ADT3_HUMAN	ADP/ATP translocase 3 OS=Homo sapiens GN=SLC25A6 PE=1 SV=4	4	0,55	0,35	3/6	50%
RAC3_HUMAN	Ras-related C3 botulinum toxin substrate 3 OS=Homo sapiens GN=RAC3 PE=1 SV=1	4	0,54	0,42	3/6	50%
TBB5_HUMAN	Tubulin beta chain OS=Homo sapiens GN=TUBB PE=1 SV=2	4	0,54	0,39	3/6	0%
TBA1A_HUMAN	Tubulin alpha-1A chain OS=Homo sapiens GN=TUBA1A PE=1 SV=1	6	0,53	0,54	5/6	0%
TBA1B_HUMAN	Tubulin alpha-1B chain OS=Homo sapiens GN=TUBA1B PE=1 SV=1	6	0,53	0,54	5/6	0%
TBB2A_HUMAN	Tubulin beta-2A chain OS=Homo sapiens GN=TUBB2A PE=1 SV=1	6	0,51	0,39	5/6	0%
GBB2_HUMAN	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 OS=Homo sapiens GN=GNB2 PE=1 SV=3	4	0,50	0,39	4/6	50%
AT1A3_HUMAN	Sodium/potassium-transporting ATPase subunit alpha-3 OS=Homo sapiens GN=ATP1A3 PE=1 SV=3	6	0,50	0,27	6/6	0%
TBAL3_HUMAN	Tubulin alpha chain-like 3 OS=Homo sapiens GN=TUBAL3 PE=1 SV=2	4	0,48	0,29	4/6	50%
G3P_HUMAN	Glyceraldehyde-3-phosphate dehydrogenase - Homo sapiens (Human)	4	0,48	0,42	4/6	0%
AT1A2_HUMAN	Sodium/potassium-transporting ATPase subunit alpha-2 OS=Homo sapiens GN=ATP1A2 PE=1 SV=1	4	0,47	0,32	4/6	50%
ATPA_HUMAN	ATP synthase subunit alpha, mitochondrial precursor - Homo sapiens (Human)	6	0,46	0,39	5/6	33%
HSP7C_HUMAN	Heat shock cognate 71 kDa protein OS=Homo sapiens GN=HSPA8 PE=1 SV=1	4	0,46	0,29	4/6	0%
SPTA2_HUMAN	Spectrin alpha chain, brain - Homo sapiens (Human)	4	0,45	0,24	4/6	0%
AT1A1_HUMAN	Sodium/potassium-transporting ATPase subunit alpha-1 OS=Homo sapiens GN=ATP1A1 PE=1 SV=1	6	0,44	0,31	6/6	0%
GBB1_HUMAN	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1 OS=Homo sapiens GN=GNB1 PE=1 SV=3	6	0,43	0,41	6/6	0%
KCRB_HUMAN	Creatine kinase B-type OS=Homo sapiens GN=CKB PE=1 SV=1	4	0,42	0,24	4/6	0%
AT1B1_HUMAN	Sodium/potassium-transporting ATPase subunit beta-1 OS=Homo sapiens GN=ATP1B1 PE=1 SV=1	4	0,30	0,42	4/6	0%

Table 4: Selection of proteins differentially expressed between PBZ and EB samples.

[a] Swiss-Prot accession number, [b] Protein name, [c] Number of observed ratios, [d] Geometric mean of the observed ratios, [e] Coefficient of variation of the observed ratios, [f] Number of patient ratio > 1.41 or < 0,71 compared to the total number of analyzed patient, [g] Percentage of ratio quantified with more than one peptide

Conclusion

The PBZ and EB have each a specific protein expression pattern. This has consequences for the interpretation of proteomic data obtained using these types of tissues as control samples. In our opinion, PBZ may be more suitable control tissue for differential proteomic analysis. However, this tissue should not be considered to be “normal” tissue and other alternatives should be sought.

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