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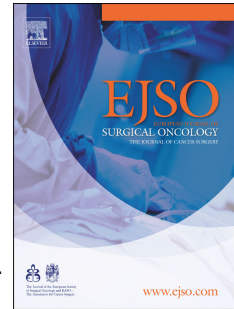
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Peptide Screening of Cerebrospinal Fluid In Patients With Glioblastoma Multiforme

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Abstract:

Aims: To apply modern mass spectrometry based technology to identify possible CSF peptide markers of glioblastoma multiforme (GBM).

Methods: Mass spectrometry based peptidomics[®] technology enables a systematic and comprehensive screening of cerebrospinal fluid (CSF) with regard to its peptide composition. Differential Peptide Display[®] (DPD) allows the identification of single marker peptides for a target disease. Using both, we analysed CSF samples of 11 patients harbouring a glioblastoma multiforme in comparison to 13 normal controls.

Results: Four CSF peptides which significantly distinguished GBM from controls in all applied statistic tests could be identified out of more than 2000 detected CSF peptides. They were specific C-terminal fragments of alpha-1-antichymotrypsin, osteopontin, and transthyretin as well as a N-terminal residue of albumin. All molecules are constituents of normal CSF, but none has previously been reported to be significantly elevated in CSF of GBM patients.

Conclusion: The study showed that peptidomics technology is able to identify possible biomarkers of neoplastic CNS disease. It remains to be determined if the identified elevated CSF peptides are specific for GBM. With regard to GBM, however, the more important role of CSF peptide biomarkers than aiding initial diagnosis might be early recognition of disease recurrence or monitoring of efficacy of adjuvant therapy protocols.

Key words:

glioblastoma, neurosurgery, cerebrospinal fluid, peptidomics, biomarker

Introduction

The peptide composition of cerebrospinal fluid (CSF) has been examined in recent years following the development of modern mass spectrometry techniques combined with suitable software solutions for multiplex analysis. Our approach in the search for marker peptides and small proteins in body fluids was termed peptidomics, defined as *„the technology for the comprehensive qualitative and quantitative description of peptides in biological samples“* [1]. The adaptation of this peptide display technology to CSF samples enables to analyze and visualize more than 6,000 peptide signals in a sample of human CSF [2]. The peptidomics approach and its performance characteristics have been published before [3, 4].

Currently there is no CSF biomarker in clinical use in malignant brain tumours, neither for diagnostic nor for follow-up purposes (like detection of recurrence or response to adjuvant therapy). The usual approach searching for CSF tumour markers is to use a technique like ELISA that is specifically directed against a particular molecule known to be involved in tumour metabolism. With regard to broader screening techniques, the proteomics approach combining 2D gel electrophoresis with mass spectrometry analysis has been applied to CSF of patients with different types of primary brain tumours [5].

The aim of this pilot study was to explore the potential of peptidomics technologies for the identification of possible peptide biomarkers of malignant gliomas in CSF. An extended knowledge of changes in CSF peptide composition could be used for the identification not only of diagnostic markers, but also of possible target pathways for therapy. Up to our knowledge this study represents the first attempt of a systematic peptide screening in CSF of patients suffering from glioblastoma multiforme.

Patients and Methods

Patient enrolment, sample collection and preparation

All CSF samples were obtained prospectively as part of a protocol for the development and evaluation of peptidomics technologies in CSF, approved by the Human Investigation Committee of Hanover Medical School (No. 1952/98). CSF was obtained for peptide analysis from tumour patients without contraindication for lumbar puncture and who gave written consent. Control lumbar CSF was acquired during the same period from patients with spinal nerve root compression syndrome undergoing a diagnostic myelography. For this pilot study we selected patients only if there were i) normal CSF cell counts, ii) normal serum lab results and iii) sterile CSF culture. According to these criteria 11 patients with GBM and 13 patients from the control group entered the study. In tumour patients the pre-operative magnetic resonance imaging (MRI) information was used to estimate the lesion volume according to the formula " $volume = height \times length \times width \times \pi / 6$ ". Furthermore tumours were described as solid, partial cystic or cystic. Uptake of contrast agent, amount of surrounding oedema and brain atrophy was graded as none, moderate, or severe.

We included CSF samples from further nine patients with various non-neoplastic conditions, resulting in different degrees of blood brain barrier disruption, to investigate the influence of blood-brain-barrier disturbance on the CSF peptide composition. The data from these samples were not used for biomarker detection but for correlating mass-spectrometric signal intensity with the function of the blood-brain-barrier.

After lumbar puncture 5 ml of CSF were immediately mixed with 0.1 ml 0.5M EDTA, centrifuged at +4°C with 2000 x g for 10 minutes and the supernatant stored at -80 °C. All samples were processed as described below.

Chromatographic separation with reversed phase HPLC

The samples were thawed gently, 550 µL CSF were diluted 1:3.75 with water and pH was

adjusted to 2-3. Using the Agilent 1100 system (Agilent Technologies, Palo Alto, CA, USA) samples were loaded onto reversed phase columns (250 x 4 mm column, source RPC, Amersham, Freiburg, Germany) and eluted with an acetonitrile gradient (4 to 80 % in 48 min) in 0.05 % trifluoroacetic acid at a flow rate of 500 μ L/min. From each sample 96 fractions (250 μ L each) were collected. The retention times of major peptide peaks were used to control the reproducibility of the method. The collected fractions were lyophilized and stored at -80 °C before further analysis.

Mass-spectrometric analysis

All 96 chromatographic fractions of a sample were analyzed with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) generating 96 mass spectra per sample. Each individual HPLC fraction was resuspended in matrix solution consisting of α -cyano-4-hydroxycinnamic acid and L-fucose (co-matrix) in 0.1 % acetonitrile/ trifluoroacetic acid (1:1 v/v). A 15 μ L equivalent of CSF in 0.3 μ L matrix was transferred onto a MALDI target. Sample ionization was performed by multi-position acquisition of 6 x 20 laser shots/spot over a representative area of the sample spot. The ions were analyzed in a time-of-flight mass spectrometer (Voyager-DE STR, Applied Biosystems, Framingham, MA, USA) in linear mode.

Peptide displays

All spectra were transferred to a database. An in-house developed software suite was used for data pre-processing and visualization, data mining and interpretation of peptide displays. This comprised baseline correction (RAZOR Library 4.0, Spectrum Square Associates, Ithaca, NY, USA), normalization of the mass spectra to a constant integral value, transformation of signal heights into color intensity for visualization and binning of spectra to 1 Dalton channels.

All 96 processed mass spectra were visualized in a 2D-gel like format, an averaged peptide display: Each peak is depicted as a bar with its colour intensity corresponding to the signal

intensity of the related mass peak. The data matrix of an individual peptide display consisted of 16 million data points. The x-, y- and z-axes represent the mass to charge ratios (m/z) within a range of 850 to 15000, the chromatographic fraction and the mass spectrometric signal intensity, respectively (Fig. 1). For all samples, an identical set of signal coordinates (fraction number and m/z value) was extracted and used for statistical analysis of the signal intensities. The underlying peptides comprise the peptidome of CSF, i.e. all peptides extracted and measured from CSF.

Differential peptide display[®] and statistical analysis

The individual datasets were combined to two master displays representing the peptidomes of GBM patients and of normal controls: Within each of the two the individual data sets were averaged for each chromatographic fraction visualized as a peptide master display. These averaged spectra were used as template for the definition of peak coordinates, which were distinct from the baseline noise. More than 6000 peak positions were identified and the signal intensities of each of these peaks were extracted into a database and entered in spreadsheets for explorative statistical analysis.

To detect possible marker candidates the two peptidomes were compared using the differential peptide display approach [6]. Subtraction maps were calculated for visual inspection and plausibility control (see Figure 1). Each measured peptide signal in the data matrix was tested for its discriminatory power by computing absolute and relative difference of the signal intensity, the Mann-Whitney-U-Test, and receiver operating characteristic (ROC) integrals. An absolute and relative difference of > 2500 units and $> 40\%$, respectively, and a ROC integral $> 80\%$ were required to reject the null hypothesis of equivalence. Peptide signals with the highest discriminatory power were subjected to identification of the amino acid sequence.

Patient data including age, duration of symptoms and the morphological MRI data were correlated to the mass-spectrometric signal intensity (SI) of the peptides in CSF (correlating with a peptide's concentration) using Spearman's rank correlation coefficient.

The latter was also used for a subset of 9 samples correlating the signal intensity (SI) to the CSF/serum albumin quotient (Q_{Alb}) and to the CSF/serum total protein quotient (Q_{Prot}).

Peptide identification by nanoESI-qTOF-MS/MS

Peptides were identified by nanoESI-qTOF-MS/MS (QSTAR pulsar, Sciex, Toronto, Canada) with subsequent protein database searches. Data processing included charge-state deconvolution and deisotoping. The resulting spectra were saved in MASCOT (Matrix Science, London, UK) generic file format and submitted to the MASCOT search engine. Cascading searches including several posttranslational modifications in Swiss-Prot (Version 39.6, www.expasy.ch) and MSDB (Version 030212 or higher, EBI, Cambridge, UK) were conducted. Results of peptide identification were accepted in automated database searches if the probability based MOWSE score for individual peptides was below a significance threshold of $p = 0.05$ indicating an absolute probability of less than 5 % for a random result from the database search.

Results

Enrolled patients

4 female and 7 male patients with a single supratentorial GBM manifestation and a tumour volume ranging from 3.6 cm³ to 96.4 cm³ were enrolled. Duration of symptoms ranged from 2-26 weeks. In the control group 4 female and 9 male patients with mostly spinal canal stenosis were enrolled. There was no difference in body mass index between the GBM and the control group, however GBM patients were significantly older than control patients (median 68 y, range 43-79 y vs. median 50 y, range 21-64 y, $p < 0.02$).

Peptide identification

Over 6000 peptide signals corresponding to an estimated number of at least 2000 different peptides were distinguished in the peptide master displays of the two peptidomes. Figure 1A shows the difference in signal intensities (SI) of the two groups. In statistical analysis eight peptide signals surpassed the indicated thresholds of significance. Five of eight candidates were successfully identified. They were specific C-terminal fragments of the proteins alpha-1-antichymotrypsin (AACT), transthyretin (TTHY), osteopontin (OPN) and an N-terminal fragment of albumin (ALB), with the abbreviations referring to these specific fragments within this article. OPN was detected twice, as single as well as double charged ion. AACT, TTHY and OPN are short C-terminal peptide residuals with 39, 28, and 65 amino acids, respectively. ALB is a 24-residue N-terminal fragment of albumin. Three candidate peptides were not identified. In two cases a glycosylation of the peptide backbone prevented the acquisition of a suitable MS/MS spectrum. A third candidate occurred with a low concentration only and no MS/MS spectrum could be acquired.

Peptide characteristics

Figure 2 shows the ROC chart for the top ranked AACT. When peptide SI in CSF was correlated to tumour volume, a negative correlation with increasing tumour volume was found for AACT ($r = -0.65/p = 0.03$). We did not observe correlations between any other

radiological feature of the tumours and SI of any of the peptides. The SI of all peptides did neither depend on direct contact of the tumour to the CSF space nor correlate to age of patients or duration of symptoms.

Correlation to blood-brain-barrier function

In five female and four male patients with non-neoplastic blood-brain-barrier disruption the SI of ALB in CSF, which corresponds to the ALB peptide concentration in the CSF sample, was correlated to the Q_{Alb} . There was a significant correlation between both parameters ($r^2 = 0.503$, $p < 0.05$). For GBM patients Q_{Alb} was not available, therefore the Q_{Prot} was used as a surrogate parameter for the barrier status. In GBM patients the SI of ALB correlated to Q_{Prot} even better as it did in the patients with non-neoplastic barrier disruption ($r^2 = 0.824$, $p < 0.01$). No correlation to Q_{Prot} was found for TTHY, AACT and OPN.

Discussion

We identified four peptides cleaved from different protein precursors that were elevated in CSF of GBM patients as compared to normal controls, thus carrying a potential as possible biomarkers in GBM diagnosis and follow-up. None of these peptides or their precursors has been reported in CSF of GBM patients so far. The presented unique approach of comparative CSF peptide profiling represents a new screening tool for analysis and visualisation of CSF peptides in an individual sample. The sensitivity of this approach was found to be as low as the mid picomolar level [3]. A comparable comprehensive profiling approach in CSF so far could only be employed on the protein level with gel-electrophoresis techniques [7] or by the combination of ultrafiltration, liquid chromatography and mass spectrometry [8].

Difference to Control group

We chose CSF of normal control patients without brain disease as a control. Only in a second step, which is beyond the scope of this paper, aspects of differential diagnosis versus other brain tumour entities can be considered. There was a distinct age difference between the tumour patients (median 68 years) and the control group (median 50 years). Therefore the higher age could have contributed to an increase in concentration of the 4 investigated peptides. The CSF proteome was analyzed with specific reference to the age of the patients/participants addressing the problem of ageing in interpretation of CSF proteomic results [9]. The authors compared older individuals with an age between 66-85 years and younger subjects aged 22-36 years. In 30 out of more than 300 identified proteins they found a greater than 20 % change in the concentration. The proteins relevant to our study (albumin, transthyretin, antichymotrypsin and osteopontin) were identified as unchanged by age. Furthermore, we correlated the signal intensities of all 4 peptides from our experiments to the age of the corresponding subjects within each group. No significant correlation was found. Thus, an age related effect on our findings seems unlikely.

Influence of blood brain barrier function

Only peptides with statistical significance in all performed statistical procedures were considered. Eight of approximately 2,000 peptides fulfilled these criteria. Therefore, a first observation is that of high stability of the CSF peptide composition in the presence of a malignant and fast growing brain tumour. The tumour induced in 10 out of 11 patients a marked surrounding brain oedema and a strong contrast enhancement following gadolinium application in magnetic resonance imaging. Both, brain oedema and contrast uptake are directly related to a defective barrier function, which leads to an increased transcellular and paracellular passage of molecules [10-12]. This increase in barrier permeability in and around a GBM, however, does not seem to lead to a significant alteration of the usual passage mechanisms for blood peptides, which occurs under normal conditions mainly by non-saturable trans-membrane diffusion or selective saturable transport systems [13, 14]. Otherwise, a more extensive change of the overall peptide composition in CSF of GBM patients should have been found.

To investigate a possible influence of the intra- or peritumoral BBB dysfunction on the results we correlated the concentration of the peptides to the extent of BBB dysfunction. In GBM, where tumour cells take up albumin in high quantities [15, 16], the increased presence of ALB in CSF could either be due to a disturbance of the BBB or due to a release of ALB or the complete albumin from GBM cells to the extracellular compartment. The first hypothesis is supported by our data. The signal intensity of ALB in tumour patients was correlated to Q_{Prot} , as this was shown for the Q_{Alb} in the group of patients with a non-neoplastic blood-brain-barrier disruption (Figure 3). The increase in CSF concentrations of TTHY, AACT and OPN seems to be independent of barrier function, since no correlation to Q_{Prot} was found.

Role of osteopontin

Osteopontin is a phosphorylated non-collageneous matrix protein, found in all body fluids and synthesized in all epithelial tissues. Osteopontin is over-expressed by almost all types

of human cancer as well as by cancer-infiltrating macrophages [17, 18]. In human malignant glioma cells and in the tumour microvasculature osteopontin gene expression and protein levels are significantly elevated [19, 20]. Osteopontin functions in cell attachment and promotes remodelling of the extracellular matrix [21, 22]. Therefore the increase of OPN in CSF of GBM patients can be best explained by a tumour-induced up-regulation of osteopontin. Neither OPN, which represents a cleavage product of a Lys-Arg cleavage site, nor osteopontin so far have been used as a tumour marker in CSF of GBM patients. OPN might not be specific for GBM, since osteopontin was reported to be significantly higher in CSF of patients with atypical teratoid/rhabdoid tumours as compared to medulloblastoma cases [23].

Role of alpha-1-antichymotrypsin

Alpha-1-antichymotrypsin is part of the acute-phase response of the host in the acute phase of inflammation, infection, malignancy and trauma. In the brain, astrocytes are a main source of alpha-1-antichymotrypsin [24]. Brain tumours, especially high-grade gliomas, can stain positively in immunohistochemistry for alpha-1-antichymotrypsin, however, this is not limited to GBM [25, 26]. There are no reports, which show alpha-1-antichymotrypsin, or a fragment of it, elevated in CSF in GBM patients. Proteases and their inhibitors are synthesized in cancer-affected tissues since they are important in tumour invasion. A correlation between the level of protease inhibitors and the prognosis was described for plasminogen activator-1 and -2 in breast cancer patients [27, 28]. Thus, an increased protease inhibitor activity in and around a malignant glioma can be reflected by an increased level of relevant cleavage products in CSF, like the identified AACT.

Role of transthyretin

Transthyretin has a function in the transport of both thyroxine and retinol in plasma and CSF. The disproportionately high concentration of soluble monomeric transthyretin (14 kDa) in human ventricular CSF is related to the fact, that it is synthesized in high

quantities within the choroid plexus cells [29] but not within the brain parenchyma. In an analysis of different brain tumours, transthyretin mRNA and protein were not found in 23 anaplastic astrocytomas and glioblastomas, but were exclusively limited to choroid plexus papillomas [30]. A dependency of TTHY to brain barrier dysfunction could not be found by correlating its concentration to Q_{Prot} as discussed above. We suspect, that a tumour-induced increase in protease activity at constant transthyretin levels is the most likely cause of the observed increase in the transthyretin fragment (TTHY).

Conclusion

This pilot study investigated the application of peptidomics analysis in the unbiased global screening for tumour-related peptides in CSF of GBM patients. The identified up-regulated peptides have not yet been reported in previous CSF studies of glioblastoma patients. They can either be attributed to tumour cell metabolism, induced changes in protease activity, induced functional changes of brain barriers, or an altered elimination. It seems possible that none of the peptides is specific for GBM. This study, however, was neither designed to validate these peptides as potential tumour markers for malignant glioma in the differential diagnosis to metastases or primary CNS lymphomas, nor investigated their utility in monitoring the response to surgery, radiotherapy or chemotherapy. The reason is that the peptidomics technique is not primarily suitable for high throughput screening in clinical routine but is designed for marker detection. The value of the peptides identified with the peptidomics approach herein needs to be determined in a trial using cheaper assays like ELISA, which are directed specifically against the identified peptides.

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References

1. Schulz-Knappe P, Zucht HD, Heine G et al. Peptidomics: the comprehensive analysis of peptides in complex biological mixtures. *Comb Chem High Throughput Screen* 2001; 4:207-17.
2. Lamerz J, Selle H, Scapozza L et al. Correlation-associated peptide networks of human cerebrospinal fluid. *Proteomics* 2005; 5:2789-98.
3. Tammen H, Hess R, Schulte I et al. Prerequisites for Peptidomic Analysis of Blood Samples: II. Analysis of Human Plasma after Oral Glucose Challenge - A Proof of Concept. *Comb Chem High Throughput Screen* 2005; 8:735-41
4. Tammen H, Schulte I, Hess R et al. Prerequisites for Peptidomic Analysis of Blood Samples: I. Evaluation of Blood Specimen Qualities and Determination of Technical Performance Characteristics. *Comb Chem High Throughput Screen* 2005; 8:725-33.
5. Zheng PP, Luider TM, Pieters R et al. Identification of tumor-related proteins by proteomic analysis of cerebrospinal fluid from patients with primary brain tumors. *J Neuropathol Exp Neurol* 2003; 62:855-62.
6. Schrader M, Schulz-Knappe P. Peptidomics technologies for human body fluids. *Trends Biotechnol* 2001; 19:S55-60.
7. Sickmann A, Dormeyer W, Wortelkamp S et al. Towards a high resolution separation of human cerebrospinal fluid. *J Chromatogr B* 2002; 770:167-96.
8. Noben JP, Dumont D, Kwasnikowska N et al. Lumbar cerebrospinal fluid proteome in multiple sclerosis: characterization by ultrafiltration, liquid chromatography, and mass spectrometry. *J Proteome Res* 2006; 5:1647-57.
9. Zhang J, Goodlett DR, Peskind ER et al. Quantitative proteomic analysis of age-related changes in human cerebrospinal fluid. *Neurobiol Aging* 2005; 26:207-27.
10. Papadopoulos MC, Saadoun S, Davies DC, Bell BA. Emerging molecular mechanisms of brain tumour oedema. *Br J Neurosurg* 2001; 15:101-8.
11. Roberts HC, Roberts TP, Brasch RC, Dillon WP. Quantitative measurement of

- microvascular permeability in human brain tumors achieved using dynamic contrast-enhanced MR imaging: correlation with histologic grade. *Am J Neuroradiol* 2000; 21:891-9.
12. Schneider SW, Ludwig T, Tatenhorst L et al. Glioblastoma cells release factors that disrupt blood-brain barrier features. *Acta Neuropathologica* 2004; 107:272-6.
 13. Banks WA, Kastin AJ. Passage of peptides across the blood-brain barrier: pathophysiological perspectives. *Life Sci* 1996; 59:1923-43.
 14. Kastin AJ, Pan W, Maness LM, Banks WA. Peptides crossing the blood-brain barrier: some unusual observations. *Brain Res* 1999; 848:96-100.
 15. Kremer P, Wunder A, Sinn H et al. Laser-induced fluorescence detection of malignant gliomas using fluorescein-labeled serum albumin: experimental and preliminary clinical results. *Neurol Res* 2000; 22:481-9.
 16. Ichioka T, Miyatake SI, Asai N et al. Enhanced detection of malignant glioma xenograft by fluorescein-human serum albumin conjugate. *Journal of Neuro-Oncology* 2004; 67:47-52.
 17. Rittling SR, Denhardt DT. Osteopontin function in pathology: Lessons from osteopontin-deficient mice. *Exp Nephrol* 1999; 7:103-13.
 18. Coppola D, Szabo M, Boulware D et al. Correlation of osteopontin protein expression and pathological stage across a wide variety of tumor histologies. *Clin Cancer Res* 2004; 10:184-90.
 19. Markert JM, Fuller CM, Gillespie GY et al. Differential gene expression profiling in human brain tumors. *Physiol Genomics* 2001; 5:21-33.
 20. Takano S, Tsuboi K, Tomono Y, Mitsui Y, Nose T. Tissue factor, osteopontin, alphavbeta3 integrin expression in microvasculature of gliomas associated with vascular endothelial growth factor expression. *Br J Cancer* 2000; 82:1967-73.
 21. Ding Q, Stewart J, Jr., Prince CW et al. Promotion of malignant astrocytoma cell migration by osteopontin expressed in the normal brain: differences in integrin signaling during cell adhesion to osteopontin versus vitronectin. *Cancer Res* 2002;

- 62:5336-43.
22. Wang DY, Yamamoto S, Hijiya N, Benveniste EN, Gladson CL. Transcriptional regulation of the human osteopontin promoter: functional analysis and DNA-protein interactions. *Oncogene* 2000; 19:5801-9.
 23. Kao CL, Chiou SH, Ho DM et al. Elevation of plasma and cerebrospinal fluid osteopontin levels in patients with atypical teratoid/rhabdoid tumor. *Am J Clin Pathol* 2005; 123:297-304.
 24. Abraham CR, Shirahama T, Potter H. Alpha 1-antichymotrypsin is associated solely with amyloid deposits containing the beta-protein. Amyloid and cell localization of alpha 1-antichymotrypsin. *Neurobiol Aging* 1990; 11:123–9.
 25. Ng HK, Lo ST. Immunostaining for alpha 1-antichymotrypsin and alpha 1-antitrypsin in gliomas. *Histopathology* 1988; 13:79-87.
 26. Kroh H, Cervosnavarro J. Alpha-1-antichymotrypsin in human glioblastoma-multiforme cells and its relation to GFAP immunostaining. *Clinical Neuropathology* 1991; 10:181-6.
 27. Foekens JA, Buessecker F, Peters HA et al. Plasminogen activator inhibitor-2: prognostic relevance in 1012 patients with primary breast cancer. *Cancer Res* 1995; 55:1423-7.
 28. Umeda T, Eguchi Y, Okino K, Kodama M, Hattori T. Cellular localization of urokinase-type plasminogen activator, its inhibitors, and their mRNAs in breast cancer tissues. *J Pathol* 1997; 183:388-97.
 29. Herbert J, Wilcox JN, Pham KT et al. Transthyretin: a choroid plexus-specific transport protein in human brain. The 1986 S. Weir Mitchell award. *Neurology* 1986; 36:900-11.
 30. Lignelid H, Collins VP, Jacobsson B. Cystatin C and transthyretin expression in normal and neoplastic tissues of the human brain and pituitary. *Acta Neuropathologica* 1997; 93:494-500.

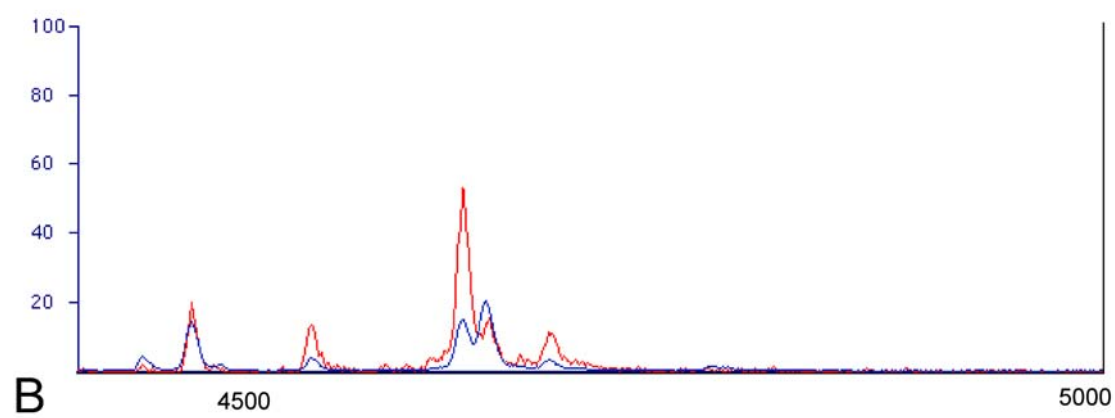
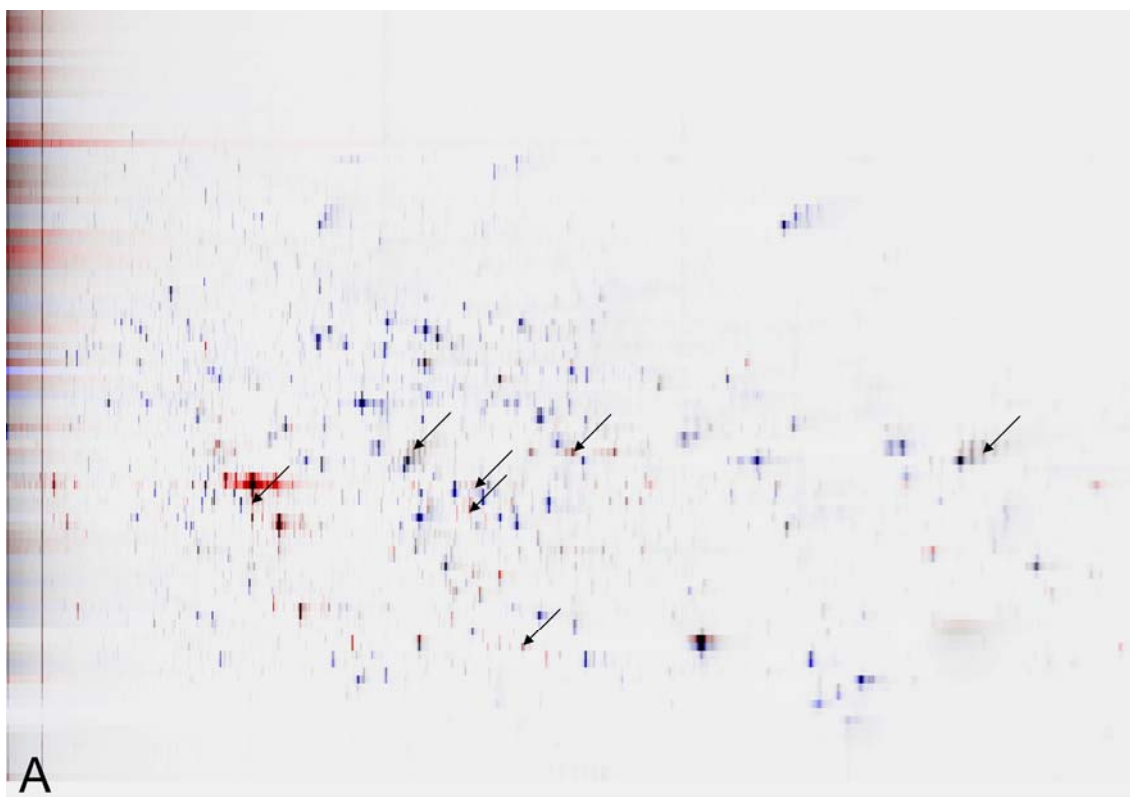
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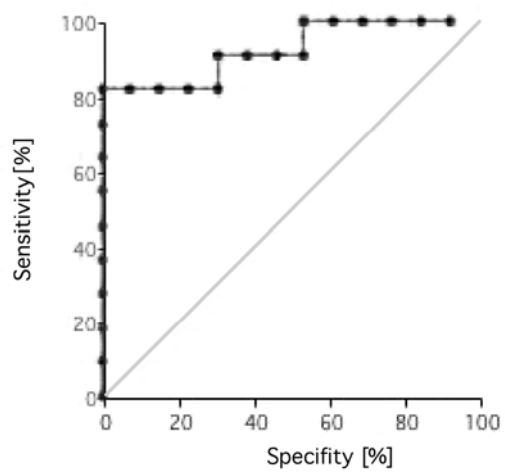
Figure Legends

Figure 1: A) The “differential peptide display” is shown, depicting the differences between the averaged (mean) signal intensities (SI) of all mass-spectrometric intensities obtained of all GBM samples in comparison with the corresponding data of the control patients. Red spots indicate peptide signals with a higher SI in the CSF of GBM patients, whereas blue spots indicate signals with a lower SI. Peptide signals, which had a comparable SI in the GBM and the control group, are depicted in grey. Arrows indicate peptide signals, which were identified by statistical testing as signals with significant discriminatory power. Not marked is the signal corresponding to the second double charged osteopontin fragment.

B) The average MALDI-TOF spectra of the fragment “AACT” from all datasets of the GBM group is plotted in red versus the corresponding data of control group in blue. The y-axis shows the SI of the peptide, which corresponds the peptides concentration in CSF. The x-axis displays the range of the relative molecular masses. Obviously, the SI of the AACT fragment is higher in the GBM group. The bottom arrow in panel A labels the peptide AACT.

Figure 2: Receiver Operating Characteristic (ROC) chart of AACT, which determines the discriminatory power (ROC area) of AACT using the ROC method, plotting sensitivity versus specificity for any given AACT value. This peptide has a high discriminatory power, since there is only a minimum loss of specificity with increasing sensitivity, resulting in a ROC integral of 92%. A sensitivity of about 82 % is given at a specificity of 100 %, with the latter one depicted as $100 - \text{specificity} = 0 \%$





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