SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Finding new ways to improve the treatment of neuro-oncological diseases

by Geza Mezey MD

Supervisor: Laszlo Bognar MD, PhD

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF NEUROSCIENCES

DEBRECEN, 2015

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By Geza Mezey, MD, Specialist in Neurosurgery

Supervisor: Laszló Bognár MD, PhD

Doctoral School of Neurosciences, University of Debrecen

Head of the **Examination Committee**:	Tamás Bíró MD, PhD, DSc
Members of the Examination Committee:	Ervin Berényi MD, PhD Tamás Járdánházy MD, PhD

The Examination takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen
23rd of September, 2015, 11 AM

Head of the **Defense Committee:**	Miklós Antal MD, PhD, DSc
Reviewers:	András Büki MD, PhD, DSc Attila Bagó MD, PhD
Members of the Defense Committee:	Ervin Berényi MD, PhD Tamás Járdánházy MD, PhD

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23rd of September, 2015, 1 PM

Antecedents of the thesis

According to definition, neuro-oncology is involved in all neoplasm affecting the central and peripheral nervous system. In the practice of neuro-oncology, tumours of the central nervous system occur more frequently than tumors of the peripheral nervous systems and treatments of the latter rather follow the principles of general oncology.

In the treatments of tumours of central nervous system many peculiar features must be taken into consideration. For this reason the treatment disciplines of tumours affecting the central nervous system are different from treatment principles of tumours of other organs. Fundamentally these distinct features of tumours of central nervous system necessitated the development of a multidisciplinary special approach, the neuro-oncology.

Therapy of tumours of the central nervous system are still based on the following three pillars:

- Neurosurgical intervention
- Chemotherapy
- Radiotherapy

All of these three pillars went through remarkable development in the past decades. Mainly the development of preoperative diagnostic has served the best achieving of maximal neurosurgical therapy. The most important improvement in preoperative diagnoses were the high resolution computer tomography (CT), magnetic resonance imaging (MRI), MRI spectrometry, functional MRI, tractography, imaging combined electrophysiological brain mapping, PET CT. These methods are also suitable to presume the histological type of the tumours besides visualizing anatomical localisation and extent. The presumed histological diagnosis can be confirm by biopsy days before the operation. Although in a case which needs urgent intervention there is not enough time to perform biopsy and wait for the results.

In the intraoperative identification of eloquent territories the development of electrophysiology and awake surgery meant improvement. Routinely used neuro-navigation helps the intraoperative spatial orientation. The cheap and repeatable ultrasound gained important role in intraoperative imaging and intraoperative CT and MRI became obtainable.

However the only chance of intraoperative histological identification still remained the intraoperative histological analysis. This analysis is the simplified hence faster version of the traditional histology. However, besides ideal circumstances and infrastructure this method provides result in approximately 30-40 minutes. This time window may be considerably long comparing to a usual neurosurgical intervention. Moreover the responsibility of this method remains under the classical, traditional histology. Additionally intraoperative histology neither gives assistance in determining the tumour borders nor shows the level of infiltration trustworthily.

The experience of the neurosurgeon has still the most important role in distinguishing healthy from tumorous tissues. Although in the past few years several alternative methods were also developed for intraoperative, in situ identification and differentiation. At last various fluorescence and radioactive labelling methods were announced to visualize the brain tumours, especially gliomas. These methods still do not give histological diagnoses and the histological diagnoses must be obtained before the labelling method because the method is not proper for any histology.

All of the labelling methods needs venous administration of the fluorescence or radioactive molecules which can accumulate in the cancerous tissue. Disadvantages of these methods are that the specificity of the labelling is low, the time window for operation is narrow and the detection of labelling necessitates specific instrumental circumstances. That is why these methods could not come into general use.

Recently some ionisation methods for mass spectrometry were proven to be potentially suitable for mass spectrometry based histological analysis. Moreover dedicated mass spectrometry based intraoperative methods were developed for analysis of tumorous infiltration in abdominal surgery. The REIMS (Rapid Evaporative Ionisation Mass Spectrometry) method is based on the electro-cauterisation of tissues, where the ions form during the electric dissection of tissues and spectrometry of those ions take place subsequently.

However in the field of neurosurgery there is no such a method which can provide fast, in vivo, intraoperative analysis and the neurosurgical special circumstances raise further difficulties to develop this method. An intraoperative instrument which can give fast histological diagnosis would greatly support the planning of the most appropriate surgical strategy. Although in surgical resection that method would be more useful which can differentiate tumour borders and healthy brain tissue also.

This kind of mass spectrometry based method would be most expedient in the surgery of gliomas, in which cases the identification of tumour borders are very difficult even for experienced surgeons. On the other hand in gliomas, especially in glioblastomas the age, the performance scores and the extent of resection remained the relevant prognostic factors and additionally the glioblastoma is the most common malignant primary brain tumour.

In cases of glioblastoma because of its infiltration potential it is well know that the total resection is practically not possible hence postoperative treatment - if the patient general state allow it - is compulsory in the knowledge of glioblastoma histology. The recent glioblastoma protocol suggests concomitant chemo-irradiation for postoperative treatment. Although the results of this recent protocol show moderate increase in average overall survival compared to the previous regimes.
However surgical excision and chemotherapy showed some improvement in the past decade, both of the extension of safe resection and the quality of postoperative treatment need further development.

Intensive researches are conducted to develop more selective and more specific chemotherapeutical agents. Genetic markers of primary glioblastomas and their role in glioma-genesis are widely investigated but a substantive molecular prognostic factor has not been identified yet. Only few therapeutically applicable molecular target were found but according to the results of their clinical trials the dramatic increase in overall survival still delays.

There is no doubt that new agents are sorely needed in the treatment of neuro-oncological diseases, especially in case of glioblastomas which could improve the outcome. Recently some of the pituitary type peptide receptors and their ligands were put in the focus of investigations. Especially GHRHR (growth hormone releasing hormone receptor) and GHRH (growth hormone releasing hormone) was investigated. Moreover selective antagonists were developed for the splicing variant of these receptors which can penetrate through the blood brain barrier. Besides the known factors in gliomagenezis these molecular markers may become prognostic factors or suitable target for therapy.

On the other hand a method which is capable to give fast, in situ, intraoperative diagnosis would gain an important role in oncological neurosurgery. This special method would be practically advantageous where the histological result determines the surgical strategy and also in case of rare diseases.

Aims

We proposed the neurosurgical adaptation of mass spectrometry in the first part of our experiments. We would like to achieve the real time, in situ, intraoperative mass spectrometry based tissue analysis where the bipolar forceps and ultrasound aspirator (CUSA - cavitron ultrasound aspiration) can be used for sampling.
Within this aim our purpose was to develop a mass spectrometry based histological analysis and intraoperative, in situ, real time differentiation of healthy and tumorous tissues. Besides above aims we would have liked to support the surgical resection and intraoperative decision making.

Therefore we investigated the identification of gliomas and glioblastomas with high priority. We also evaluated the differentiating potential of our method between gliomas and peritumoral tissues.

Moreover we studied the other possibilities in our method such as identification of lipid fractions and peptides. We hypothesised that identification of peptide or protein fragments later enables the determination of specific molecular markers with the help of mass spectrometry.

The lack of a molecular prognostic factor or a therapeutic target is especially perceptible in case of gliomas and in the postoperative treatment of glioblastomas also. Therefore in the other part of our experiments we aimed to study a usable prognostic factor and molecular target with conventional molecular genetic tools for the present. We investigated the ligand GHRH, pituitary type GHRH receptor and its splicing variant receptors as prognostic and therapeutic targets in human glioblastoma samples alongside the proven role-playing EGFR and PTEN genes.

Moreover our studies showed that our mass spectrometry based method could play a role in the fast identification of rare tumours. Hence in our dissertation we present a rare disease, the epidural lymphomas in which our results proved that if a fast, intraoperative histology would be available during the operation this histological result can determine the surgical strategy.

Materials and methods

Combination of the bipolar forceps and mass spectrometry (BF-MS)

Commercially available bipolar forceps with irrigation was used (ERBE Elektromedizin GmbH, Tübingen, Germany) for ionization during *in-vivo*, human, surgical interventions. The ionization of the sample takes place at the surgical site during the electrosurgical dissection of the tissue pieces similarly to the REIMS method. The generated surgical smoke is lead away through the gap of the forceps originally created for irrigation. A 4 meter long, 1/8” diameter Teflon tube is mounted on the other end of the forceps and connected through a micro air jet pump-based ion transfer apparatus or directly to the home-made capillary inlet of an LTQ Velos (ThermoScientific, Bremen, Germany) or an LCQ Deca XP Plus (ThermoFinnigan, San Jose, CA, USA) mass

spectrometer. The micro pump was driven by pressurized air (from the central pressurized air system of the hospital) at a nominal inlet pressure of 2.5 bars. The pump was installed into a commercial electrospray ion source of LTQ Velos and positioned orthogonal to the capillary inlet of the atmospheric interface in order to reduce the amount of contamination reaching the inner part of the mass spectrometer.

Course of intraoperativ, in situ studies with the bipolar forceps coupled mass spectrometer and ethics

In our experiments we always used the above introduced bipolar forceps coupled mass spectrometer. The computer, the database and the used software were integrated into our compact system. All parts of the system were totally adapted to the operative and intraoperativ circumstances.

Important to note that during the operations we performed routine resections of tumours and coagulations of tumors surfaces which commonly happen through resection and the electro-thermic effect of coagulation always yields to the evaporation of small amount of the tumour tissue.

Coagulations during the corticotomy or on white matters always served the tumour resection. In some cases we removed additional peritumoral tissue for histological verification but the resected peritumoral cortex or white matter would have been resected or sucked out anyway. The spectrum of the above mentioned territories were recorded as peritumoral brain tissues. In every cases we only used the normally arisen aerosol for the study. The intraoperative investigation was authorized by the Ethics Committee of University of Debrecen.

Spectrometric data of tumours coagulated and evaporated by bipolar forceps was collected during 44 surgical interventions. Moreover in 4 cases we could record the spectrums of peritumoral tissues which were coagulated from necessary intraoperative reasons. The collected tissue specific database was tested with operation of 11 additional tumour.

After the operations the resected tumours were histologically analysed orderly, and invariably from the local regulations. Tumour parts were not excised from the resected tumours. Histology was performed in Department of Pathology, University of Debrecen or University of Giessen.

The recording and storage of the spectrums were the same as we used in experiments of CUSA coupled mass spectrometry.

Combination of CUSA and mass spectrometry (CUSA/SSI-MS)

A commercially available ultrasonic surgical unit (Selector, Erbe Elektromedizin GmbH, Tübingen, Germany) was used for tissue disintegration and removal. Ultrasonic disintegration was performed at 24 kHz, with a 60% energy setting. PTFE tubing (1/16 in. o.d., 1.2 mm i.d.) was placed into the drain line of the commercial surgical hand piece in order to minimize memory effects. This tubing was connected to a custom-built Venturi pump through a 2 m long (1/8 in. o.d., 2 mm i.d.) tube. The Venturi pump was driven by nitrogen with the inlet pressure set to 10 bar. The Venturi pump was mounted on the mass spectrometer atmospheric interface orthogonal to the heated capillary inlet. An identical atmospheric interface setup was described earlier termed Venturi easy ambient sonic-spray ionization (V-EASI). High-resolution mass spectrometry was performed using a Thermo LTQ Orbitrap Discovery instrument (ThermoScientific, Bremen, Germany). Low-resolution

measurements were performed using an LCQ Deca XP Max quadrupole ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). During regular ultrasonic tissue resection, a vibrating hand piece is brought into the proximity of the tissue surface and the vibration (~20-40 kHz) is transmitted to the tissue by an annular water jet. The liquid is continuously removed from the tissue surface by vacuum suction through a hollow hand piece with typical flow rates in the range of 2-10 ml/min.

Course of intraoperativ, in situ studies with the bipolar forceps coupled mass spectrometer, ethics

In our experiments we always used the above introduced CUSA coupled mass spectrometer. The computer, the database and the used software were integrated into our compact system. All parts of the system and also the whole system was totally adapted to the operative and intraoperativ circumstances.

At first food grade porcine organs were used for the systematic characterization of experimental parameters.

For the ex vivo experiments human brain tumour samples were obtained from the Institute of Neurosurgery, University of Debrecen (Debrecen, Hungary) and Klinik fur Neurochirurgie, Universitatsklinikum (Giessen and Marburg, Germany). Human brain slices samples were obtained from the Institute of Pathology, University of Debrecen. Complete ethical clearance was obtained for the collection and analysis of human samples.

Data Analysis and storage of the spectras collected by bipolar forceps or CUSA coupled mass spectrometer

Mass spectra were collected in single stage MS, negative and positive ion mode, in the mass range 150-1000 m/z, unless otherwise stated. Spectral data was binned using a 0.01 m/z bin size in the case of high-resolution experiments and a 1.0 m/z bin size in the case of low-resolution experiments and stored in an SQL database (Oracle, Redwood City, CA) containing the full known classification of every tissue specimen corresponding to the spectra, including WHO tumour type and grade. On a chosen, normalized subset of spectra, principal component analysis (PCA) was carried out in order to reduce the dimensions to 60. PCA was followed by linear discriminant analysis (LDA) without further reduction of the dimensions. The number of components was selected based on a previously described cross-validation study where the misclassification rate was tested as a function of the number of components. The resulting PCA/LDA model was saved in the database and used in the tissue identification process or, alternatively, the points were plotted as function of the first 3 PCA or LDA components for demonstration purposes. The real-time classification of new spectral data is based on the saved PCA/LDA model, the new spectrum is first transformed to the 60-dimensional PCA space then to the 60-dimensional LDA space, and subsequently a squared Mahalanobis distance is calculated to every class average in the model. The new spectrum is classified to the closest class average, if the spectra is in the range of ± 5 times the standard deviation from the class average in every dimension. If the spectrum is outside of the above specified range for all included tissue classes, the spectrum is marked as “outlier”.

Aerosol Inhalation Safety Regulations

An appropriate breathing mask (M7500 series mask with 6055 A2 filter, 3 M Deutschland GmbH, Neuss, Germany) or regular surgical mask with same parameters was worn throughout all experiments.

Sample collection of genetic experiments, ethics

Twenty-six tissue samples (23 glioblastomas and 3 peritumoral tissues) were collected during neurosurgical operations in the Department of Neurosurgery, University of Debrecen, Hungary. The samples were frozen immediately after removal in liquid nitrogen and stored at −80 °C until processing. Sections were cut from the same samples for histological analysis and genetic investigations. Lesions were diagnosed on the basis of formalin-fixed paraffin embedded (FFPE) tissue sections by haematoxylin-eosin staining and by additional immunohistochemistry. Classification of tumors was based on the World Health Organization International Classification of Tumours 2007. The local institutional ethics committee approved the collection and use of these specimens for the current study and informed consent was obtained from all patients.

RNA extraction and RT-PCR (reverse transcriptase - polymerase chain reaction) for detecting GHRH, pGHRHR, SV1 and SV2.

Frozen tissue samples were incubated overnight at −20 °C in RNAlater Ice solution (Qiagen, GmbH, Germany). RNeasy mini kit (Qiagen, GmbH, Germany) was used to isolate total RNA from tissue samples. RNA quantity and quality were determined by NanoDrop ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and 1 % TAE agarose gel. Two hundred fifty nanograms of RNA from each sample were reverse transcribed into cDNA by QuantiTect Reverse Transcription kit (Qiagen) in a final volume of 20 µl. The integrity of cDNA was tested by RT-PCR for β-actin (ACTB). Gene-specific primers for pGHRH-R (sense: 5′-CACGTCTTCTGCGTGTTGAG-3′, antisense: 5′-GCATCTCCTCTGCTGCTTGT-3′) and SV1 (sense: 5′ GGAGTTGTGGCTAGAGAG-3′, antisense: 5′-GCATAGAACAGTGGAGAAG-3′) were designed with the primer3_www.cgi v0.2 program, and primers for GHRH and ACTB were described previously. In all RT-PCR reactions, 1 µl of cDNA was amplified in a 25 µl solution containing 1.5 mM MgCl2, 1× PCR buffer (Fermentas, Germany), 0.3 mM of each deoxynucleotide (Promega, Germany), 1 unit of TrueStart HotStart DNA polymerase (Fermentas) and 0.25 µM of each primer. Samples were denatured for 3 min at 95 °C, then subjected to 30 (ACTB) or 40 cycles of 95 °C for 45 s, 54 °C (SV1), 59 °C (SV2) 64 °C (GHRH) or 67 °C (pGHRHR) for 30 s, then 72 °C for 1.5 min with a final extension of 10 min at 72 °C; 10 µl of each amplification reaction was electrophoretically separated on 1.5 % agarose gel, stained with ethidium bromide, and visualized under UV light.

Quantitative real-time RT-PCR for detecting EGFR and PTEN expression

Six hundred nanogram of RNA from each sample was translated into cDNA applying High Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA, USA) according to the manufacturer's instructions. SYBR Green Premix Ex Taq-based assay was used to determine the mRNA expression level of EGFR and PTEN genes (Takara Bio Inc., Japan). For the analysis, the following primers were used,

each in 0.25 μM final concentration: EGFR (forward: 5′-CTC AGC CAC CCA TAT GTA CC-3′; reverse: 5′-CGT CCA TGT CTT CTT CAT CC-3′), PTEN (forward: 5′-AGC ATC ACC ATT CTT TGC TG-3′; reverse: 5′-ACC ACA GCC ATC GTT ATG AA 3′) and GAPDH (forward: 5′-CAG TCA ACG GAT TTG GTC GT-3′; reverse: 5′ TTG ATT TTG GAG GGA TCT CG-3′). Each reaction contained 15 ng cDNA and was run in triplicate on M×3005P™ real-time PCR system (Agilent Technologies, USA) with the following thermal profile: activation at 95 °C for 1 min; 40 cycles of amplification at 95 °C for 1 min, at 55 °C (GAPDH), 58 °C (EGFR) or 60 °C (PTEN) for 30 s followed by 72 °C for 1 min. Melting curve analysis was also performed in each case. Fold changes in gene expression were calculated by the ΔCt method **(55)** using GAPDH as internal control and peritumoral tissue sample as calibrator.

Statistical analysis for the gene expressions and clinical data

For statistical analysis, the IBM SPSS Statistics 19 software (IBM Corporation, USA) was used. In cases of EGFR and PTEN, more than twofold change in the gene expression level was considered as gene down- or up-regulation. On the basis of the Kolmogorov–Smirnov test, the distribution of EGFR expression data is significantly different from a normal one so nonparametric tests were applied to compare the mRNA expression levels of EGFR (Mann–Whitney U test), PTEN (independent samples median test) genes and the expression profiles of GHRH, SV1 and clinico-pathological data. Kaplan–Meier method with log rank (Mantel– Cox) test was used for survival analysis. Differences with $p < 0.05$ were considered statistically significant.

Methods for the analysis of spinal epidural lymphomas

We collected and retrospectively evaluated the clinical data of 1166 patients treated with lymphoma by the Department of Internal Medicine and Department of Neurosurgery in the last two decades. Clinico-pathological, diagnostic and therapeutic information of 512 Hodgkin lymphoma and 654 Non-Hodgkin lymphoma cases were revised. In 13 cases of the disease was accompanied with epidural mass effect or emerged as epidural process firstly.

Results

Our result with bipolar forceps coupled mass spectrometer

Spectrometry was performed during 55 operations. During operations of the first 44 patient we recorded 264 spectras and we built up our tissue specific database. Histology of these 44 tumors were glioblastoma, gliomas, metastases, meningeomas, lymphomas, neurilemmomas, gangliogliomas. In 4 cases of 44 we could sample peritumoral brain also. The tissue specific database was tested on 11 additional patients with 47 entries of 5 glioblastoma multiforme, 3 metastasis with 1 peritumoral and 3 meningioma in addition to the cross validation of the previously built PCA+LDA models.

The spectral species in both tissues mostly overlap, however the ratio of each characteristic species is significantly different and show a tissue specific distribution. As previously expected, the peak distribution in metastatic cancer tissues differ more

from the brain tissues than the primary brain cancer tissues compared to peritumoral, healthy brain tissue. However, there is a significant difference between the healthy and cancerous brain tissue, and the separation of all tissues using PCA+LDA algorithm has proven to be successful.

Even though 55 patients were recruited, the diversity of the brain cancers makes it difficult to collect enough data for multivariate statistics. However, our algorithm using multivariate statistical data processing is capable of separating healthy and cancerous brain tissue, primary and metastatic brain tumors and also has a potential in separation of different grade primary brain tumors.

The 55 patients were divided into two groups, where the spectra of the first group containing 44 patient was uploaded to the database, while the second group containing spectra of 11 patients was used as an independent validation set.
Especially the validation results of glioblastomas, meningeomas and metastases showed unexpected results with 100% validation. There is no doubt that this good results originated from the small number of recruited patients and the comparison was between 264 database and 47 validating spectras.

As only one patient was recruited with ganglioglioma and one patient with lymphoma, the spectra of these tumour types were only plotted on PCA and LDA plots for illustration; cross-validation and independent validation was not possible.

We performed the leave-one-patient out cross-validation using the peritumoral brain spectra and cancerous brain spectra. Although histological categories had to be merged in order to reach enough number of patients and spectra in each category, significant sensitivity could be observed in the identification of peritumoral tissues.

The advantage of this method in our experiments was mainly that the aerosol produced by the coagulation could be directly used for mass spectrometry. On the other hand we could utilize our experiences in the REIMS method.

Nevertheless the most important disadvantage of this system was the same electro-thermic effect based evaporation and sampling which could be apply in neurosurgery limitedly, especially if we would like to examine not only the tumour itself. Near eloquent territories CUSA method seemed to be more suitable for sampling.

Our result with CUSA coupled mass spectrometer

Ultrasonic aspirator devices disintegrate tissues and evacuate the resulting tissue debris. Because of the continuous flushing of the surgical site, the tissue debris is continuously drained away in form of a dilute suspension in physiological saline. Theoretically, the online mass spectrometric analysis of this drainage could provide sufficient data for the identification of the disintegrated and aspirated tissues.

Although spray ionization of the drainage seems to be a straightforward approach, the presence of macroscopic tissue particles effectively hinders the utilization of any traditional spray setup that employs capillaries as a mean of liquid transfer. The recently described Venturi easy ambient sonic spray ionization technique, however, allows the use of tubing with diameters exceeding 1 mm. A further advantage of the Venturi device is that it provides sufficient suction force for the liquid transfer. The experimental setup was tested using porcine brain samples as a model system of in situ, in vivo system.

The effect of various instrumental parameters on spectral intensity was investigated, including the power setting of the ultrasonic surgical device, nitrogen inlet pressure of the Venturi-pump, relative geometry of the atmospheric interface, voltage settings of the ion optics, and inlet capillary temperature. Signal intensity is strongly dependent on the ultrasound power. Results clearly indicate that ultrasonic disruption of the cells is a prerequisite for obtaining high concentrations of cell components in the draining liquid and thus sufficiently good spectral quality. The data also show that the presented strategy cannot be used in the absence of ultrasonic disintegration, i.e. in the case of simple surgical aspirators which are also widely used in neurosurgery.

Investigation of the dependence of signal intensity on the nitrogen inlet pressure of the Venturi pump shows a dynamic relationship, with a clear saturation phenomenon in the 0-30 bar range. Because of the clear optimum value, a 10 bar nitrogen inlet pressure was used throughout the experiments described below. Both the dependence of signal intensity on the nebulizing gas flow rate and the overall experimental setup suggest a sonic spray-like ionization phenomenon, since the sample undergoes a solely pneumatic spraying, in the absence of electric potential gradients or thermal effects. This assumption is also in agreement with the observation that only species undergoing dissociation in the liquid phase are detected. In order to provide experimental support for this hypothesis of sonic spray-like ionization mechanism, filtered tissue homogenate was analysed by using a traditional sonic spray ion source. The resulting spectra were highly similar to the CUSA/SSI. Further experimental evidence was provided by spraying amino acid glutamine at different pH values. The pH dependence of the intensity of ions were almost identical using the Venturi device and a more traditional sonic spray source and both followed well the theoretical speciation of this amino acid, similar to earlier studies.

The relative position of the Venturi pump and the atmospheric interface also has a dramatic influence on signal intensity. Optimal signal was obtained when the central axis of the Venturi pump pointed toward the inlet opening. The distance was found to have an optimum at ~20-25 mm. Although the angle of the Venturi pump and the ion optics only moderately affects the actual signal intensity, the orthogonal setup was chosen in order to minimize the contamination of the instrument.

The influence of the inlet capillary temperature on the signal intensity of phospholipid compounds was also investigated. Under 100 ^{0}C, the intensity of phosphatidyl-inositol 38:4 was negligible; however, from 100 to 400 ^{0}C an increasing tendency was found. Since ionization efficiency improves with increasing temperature, the result is also in agreement with the sonic spray-like ionization phenomenon. The probability of obstruction of the inlet capillary by sticking tissue parts also increases with higher temperature; therefore, 250 ^{0}C was chosen as a compromise.

Optimization of the instrumental parameters (i.e., relative position of the Venturi pump, inlet capillary temperature) allows the continuous operation of the CUSA/SSI-MS system for longer than 4h. In comparison, during a 2-3h long surgical intervention, the ultrasonic aspirator is used overall for 20-30 min.

Possible obstruction of the CUSA transfer tubes is already well known in the surgical practice; therefore, the assistance personnel is prepared for the flushing of the system. The memory effect of the ion source was also tested, and no significant carry over was observed.

Optimal settings of the ion optics for the Venturi-sonic spray ionization technique were considerably different from the optimal settings for electrospray

ionization (ESI) or atmospheric pressure chemical ionization (APCI) ionization. Direct current potentials applied onto the inlet capillary or the tube lens had negligible influence on the signal intensity; however, floating the skimmer electrode at -90 V resulted in an approximately 5-fold signal enhancement.

The general sensitivity of the method was determined regarding both full spectral information and the detection of individual compounds. In the former case, various amounts of porcine brain white matter tissue were suspended in 1 mL of saline by the ultrasonic surgical hand piece and aspirated by the Venturi pump device. The resulting spectra were integrated, and the signal-to-noise ratio was plotted for various peaks as a function of suspended tissue weight. It is concluded that full spectral information can be obtained using 500 μg of tissue or more, while the spectral pattern remains unchanged. Major peaks, however, were detected using as small as 50 μg of tissue sample suspended in 1 mL of liquid. Sensitivity to individual compounds was tested using Substance P and phosphatidyl ethanolamine (15:0/16:0). Because of the presence of an odd-numbered acyl chain, the natural concentration of this lipid species is very low. The limit of detection and dynamic range were tested at three different brain tissue concentrations. The observed LOD values (corresponding to a 3:1 signal-to noise ratio) were in the low nanogram/millilitre range for the peptide species and in the 100 ng/mL range for the phospholipid compound. These sensitivity values are comparable to those of conventional sonic spray ionization and allow the detection of tissue components at trace concentration levels.

As it was expected upon theoretical considerations, the spray ionization of tissue debris produces predominantly ions corresponding to various lipid species ranging from simple fatty acids to glycerophospholipids or sulfatide glycosphingolipids. Analysing porcine brain samples we could identify different lipid species.

Unlike other “ambient” mass spectrometric methods like REIMS, the cavitron ultrasonic surgical aspiration/sonic spray ionization (CUSA/SSI) method produces ions of metabolic constituents, carbohydrates, and peptides besides the highly intensive lipid-related ion population.

Multiply charged peptide anions have also been observed in the negative mode. The ion signals indicate the presence of species A with a molecular mass of 3788.3 Da and peptide B with molecular mass of 4960.5 Da. Species A was associated with calcitonin gene related peptide (CGRP), which is an abundant peptide in the CNS and has been described as a diagnostic marker in the case of lung tumors. Species B was tentatively associated with thymosine β 4 peptide. Thymosine β peptide has also been described as a prognostic marker in the case of non-small cell lung cancer (NSCLC). Our tentative identification is based on the facts that the sample was a brain metastasis of NSCLC, and the corresponding peptides have been associated with metastasis formation; however, further experiments are needed for the validation of these identifications.

The main objective of the present study was to develop a mass spectrometry-based technique for the intraoperative identification of tissues, which aids the surgeons in removing all malignant tumour tissues while the healthy tissues are preserved. Providing tissue specific data is a prerequisite in this regard for the method. The CUSA/SSI spectra show considerable tissue specificity even in the case of healthy brain samples. Spectra collected from the grey and white matters show characteristic differences.

Similar to the complex lipid data collected by matrix-assisted laser desorption ionization (MALDI) imaging, desorption electrospray ionization-mass spectrometry imaging (DESI) imaging or REIMS techniques, the tissue specificity of the spectra is generally not associated with tissue-specific biomarkers, rather due to the different distribution pattern of a similar set of lipid species. Since individual ion intensities are less informative, we chose to utilize full spectral information for identification. Obviously, identification using mass spectral "fingerprint" requires a database of histologically assigned, authentic spectra. The spectral database was constructed using fresh, ex vivo samples and fresh post-mortem samples. Spectral differences between ex vivo and post-mortem samples (within 24 h following exitus) were found negligible, using different animal models.

A database comprising 284 histologically assigned spectra of human brain tissues and brain tumors was used to develop an identification algorithm. Mass spectra recorded in the m/z 600-1000 were binned and normalized to an integrated total ion count. Appropriate subsets of the database were subjected to principal component analysis in order to decrease the number of dimensions to 60, similar to previously reported applications.

Although the data already shows complete separation, supervised linear discriminant analysis was also performed to enhance the segregation of the data groups. The result of the LDA, obviously using only the first 3 LDA parameters can be depicted in co-ordinate system. Hence, the proposed quasi-real time data analysis comprises the localization of the actual spectrum in the 60-dimensional LDA space and its classification into the closest histology-specific data group. Since the LDA space is nonorthogonal, the squared Mahalanobis distance function is used as a metric. The time demand for the identification of unknown spectra is <0.1 s with a precalculated PCA/LDA map, which is negligible compared to the 2-3 s overall analysis time. Identification performance for glioblastoma multiforme and healthy brain samples were 100%; however, this unexpectedly good performance was obtained partially due to a limited number of samples, and it has to be refined in further studies using subject numbers in the order of a few hundred to one thousand.

Moreover the hand piece of CUSA was successfully registered to our neuro-navigation system earlier. That's why the spatial orientation of the sampling similarly to the bipolar forceps easily is reproducible and the radiological parameters become precisely comparable with the mass spectral information.

Clinical course of the patients operated with glioblastomas

Twenty-three patients suffering from primary GB were operated in our department during the period of November 2005 and November 2008. Samples from their resected solid tumors were studied. The largest tumour diameter was >3 centimetres in all cases. In case of 15 patients, the operation was performed at the first recognition of the tumour. After surgery, standard postoperative treatment was initiated. The postoperative treatment was cancelled in four cases because of the rapid progression of the tumour and, in one case, because of non-compliance by the patient. Eight of the 23 patients had previous surgery and received regular postoperative treatment for proven GB histology. In these cases, samples were collected at the reoperation of the tumour recurrence. In four of these cases, we also had samples from the first operation. In 3 cases, we obtained peritumoral tissues, which required removal to facilitate approach to the tumors themselves. These tissues were used for

calibration in subsequently measuring EGFR and PTEN expression levels. The mean age of the patients sampled was 59.8 years ± 2.2 SE (range 37–78 years), with 14:9 ratio of female to male. The follow-up lasted until the death of the last patient (June 2011). Mean overall survival was 536.3 days ± 107.0 SE (n = 19) from diagnosis, and mean survival from sampling was 411.3 days ± 90.3 SE (n = 23). The four operated and later repeated patients were calculated, of course, as one patient in the relevant statistics. All of the patients died because of tumour progression. Clinicopathological data of patients were meticulously collected.

Expression of pGHRHR, SV1, SV2 and GHRH in human GB samples

Pituitary-type GHRH (pGHRHR) receptor was not expressed in our sample set while SV1 could be detected in 17.4 % of the tissues (4 out of 23). Another—presumably non-functional—splice variant, SV2, could be detected in 2 samples (8.6 %). To examine the possible autocrine and paracrine effect of the natural ligand, the expression of GHRH was also determined and found to be positive in 14 samples out of 23 (61.9 %). Only one sample expressed both the ligand GHRH and receptor SV1. Among the 9 of 23 GHRH negative samples, three were positive for SV1.

Expression levels of EGFR and PTEN

The expression levels of EGFR and PTEN genes were presented as normalized data as it was written in the "Materials and methods" section. In 15 of 23 samples (65.2 %), significant EGFR over-expression could be detected, and 18 samples showed significant PTEN gene under-representation (78.3 %). In case of 11 patients (47.8 %), EGFR up-regulation was accompanied by PTEN down-regulation.

Expression profiles of samples from the same patient prior and after therapy

In case of 4 patients, we had the opportunity to take tumour samples both at the first operation and at surgery for recurrence. In 3 of these cases, GHRH was present in the initial tumour but could not be detected in the tumour tissue from the recurrence. SV1 was present in only one tumour at the time of first operation. EGFR and PTEN showed normal expression in only one tumour but also in this case, EGFR was overexpressed and PTEN was down-regulated in the tissue of the recurrence. Otherwise, EGFR mRNA was over-represented in all samples and PTEN was down-regulated in all but one recurrent tumour tissue.

Analyses of gene expressions and clinico-pathological data

Thorough statistical analyses of the studied expression levels we found that in SV1 positive samples the PTEN expressions showed significantly increased levels compared to SV1 negative cases. For further analysis, we divided the patient into two groups. Group 1 included 15 patients who were operated at clinical diagnosis, without previous treatment. Group 2 included 8 patients where the recurrent tumour was sampled after chemo-irradiation. Four patients were present in both groups as operated and later reoperated cases. In Group 1, patients with GHRH negative tumors had significantly decreased overall survival (OS). This decrease remained significant even in the presence of SV1 positivity, which showed connection with higher median levels of PTEN. In Group 2, we did not find these correlations. Analysis of OS in the

two groups together is questionable because the overlapping four patients showed that the therapy and tumour progression might lead to changed genetic profile. However, analysis of OS in the combined two groups (excluding the four overlapping patients in the second group) also revealed that the GHRH negative cases showed shorter OS, which was still significantly decreased in cases that were GHRH negative and SV1 positive. Conversely, GHRH positive and SV1 negative cases showed better prognosis. EGFR and PTEN expression levels were not correlated significantly with OS. Although, the lack of postoperative treatment itself seemed to lead to shorter survival time, in these cases rapid tumour progression produced a decrease in KPS (Karnofsky Performance Score) and led to cancellation of the suitable therapy (which should have been used postoperatively).

Role of mass spectrometry based real time, in situ histology in the treatment of epidural malignant lymphomas

We found 13 epidural involvement (7 Hodgkin and 6 Non-Hodgkin lymphoma) in the evaluated period. This incidence is comparable with occurrence published in the literature. In 6 of 13 (46, 15%) cases the first presentations were in the epidural mass and the postoperative histology revealed the lymphoma diagnosis. All 6 patient had postoperative treatment. Although we performed just decompression in all cases and there were no sign of instability the postoperative course of these patient shows well that the intraoperatively known lymphoma histology press the surgical strategy toward the minimally invasive decompression. Moreover if instability can be observed even primary or secondary to the operation the long overall survival suggests spinal fixation for maximal preservation of mobility.

Discussion

The first aim of this thesis was the development of a tool based on mass spectrometry which is suitable for in situ, in vivo tissue identification. Aerosol produced by bipolar electrothermic coagulation had the advantage of direct suitability to spectrometric analysis. The method was also proved to be suitable for fast intraoperative histological diagnoses but the electrothermic effect reduced the utilization. Therefore our interest turned toward the delicate suction methods.

Recently in neurosurgery the smallest tissue parts can be removed by the CUSA without destruction of surrounding structures. Though in this case the sample is not suitable immediately for mass spectrometry, it must be transformed to gas phase and ionized. This was solved by inserting a Venturi pump into the athmosferic interface of the mass spectrometer. This CUSA coupled mass spectrometer needed several test experiments to ensure the optimal settings.

Ex vivo, but within surgical conditions we showed that the system is suitable for selective tissue compounds analysis. Moreover we successfully identified peptid receptors with our system.

The presented data clearly demonstrates that mass spectrometric analysis of liquefied tissue debris produced ultrasonic ablation has the potential to provide meaningful data for the identification of the corresponding tissues. The method has been tested for the analysis of ex-vivo samples in the operating room. It was shown to provide already considerably faster tissue identification (2-3 s versus 30-40 min) than frozen section-based intraoperative histology or off-line mass spectrometric analysis of tissue specimens collected by a CUSA device (~10 min).

Our analytical results were especially successful in case of gliomas, glioblastomas and metastatic tumours. Although with appropriate database other types of tumours can be identified.

The method can be especially useful in case of rare lesions which may need acute intervention and where the intraoperative histological diagnoses can change the surgical strategy. To illustrate this we presented the retrospective analyses of our spinal epidural cases. It is well known that the chemotherapy and radiotherapy of lymphomas are exceptionally efficient. That is why if an intraoperative histology would be able to reveal the lymphoma diagnosis that diagnosis would suggest the minimal invasive solution instead of attempting total tumour removal

Further advantage of online analysis is that in principle all the aspirated tissue material can be subjected to identification this way, which may significantly improve the precision of the intervention. Although the method has not yet been tested for in vivo tissue identification, the method is expected to perform similarly, especially since ultrasonic tissue ablation does not induce extensive bleeding, thus the drain sample does not contain an excessive amount of blood. Besides of all we showed that the system can make difference between healthy and tumorous brain tissues and this would be particularly useful in case of infiltrating gliomas. In a broader context, ultrasonic ablation directly coupled with spray ionization can also be utilized as a general surface analysis technique, similar to the surface sampling probe-electrospray mass spectrometry (SSP-ES-MS) technique described by Van Berkel et al.

We introduced that our method can help the resection of gliomas and glioblastomas, but in case of glioblastomas even after macroscopically total resection the disease practically always recurs. In postoperative treatment of glioblastomas the results of current protocols still do not show outstanding breakthrough. There is no doubt that further investigation of molecular targets and prognostic factors of glioblastomas are sorely needed.

Our study provides the first evidence that GHRH and SV1 are present in a substantial part of human GB tissues. Moreover we pointed out that the expression profile of GHRH and SV1 correlates to the overall survival. The expression of GHRH was determined and found to be positive in 61.9 % of the samples which suggests that GHRH has a role in the pathogenesis of GB. This is supported by previous studies, which reported improved cell survival of cardiac or tumoral cells following treatment with GHRH. Substantial statistical analysis of our clinico-pathological data revealed a significantly negative correlation between prognoses and the lack of GHRH expression, and this remained significant if SV1 were also expressed. Conversely, GHRH positive, SV1 negative cases showed better overall survival. It is partly supported by Farkas et al. who previously showed that the expression of GHRH-R was correlated with a poor response of rectal cancers to chemotherapy. This finding also suggests that not only paracrine and autocrine GHRH release could play a role in tumour progression through SV1 receptor activation and not only local intratumoral regulating factors may decrease the expression of tumoral GHRH. The tumorous GHRH expression, i.e., autocrine release of GHRH might be under influence of negative feedback effect. This effect may be based on down-regulation of GHRH expression through SV1 or other receptors mediated by systemic GHRH or other ligand. GHRH may signal through another receptor than SV1 and it could be protective. One of the presumed receptors that could mediate the responses to GHRH might be receptors for VIP (vasoactive intestinal peptide), and these receptors might be protective as in the case of inhibitory effects of VIP on the growth of lung cancer.

Renal cell carcinoma was also reported by Vacas et al. to be inhibited by VIP and PACAP (pituitary adenylate cyclase-activating peptide).

In brief the further investigation of GHRH pathway can lead to therapeutic results.

New scientific results of the thesis

In my dissertation I presented that we successfully combined bipolar forceps and ultrasonic aspirator with mobile mass spectrometer. In this manner we created a tool not only for a real time but also for in situ tissue analysis. Moreover this method has the fundamental advantage of mass spectrometry namely the ability to analyse molecular constitution of tissues. This molecular description may open further gates by allowing the comparison of the mass spectrum to the presence of molecular markers.

In this way real time tissue analysis can also help the decision making process between forced resection or ending the operation when total resection is pointless and radiotherapy and/or chemotherapy may be more beneficial. Therefore in this thesis we showed that retrospective analysis of a rare entity could reveal the importance of real-time intra-operative histology.

Moreover in this thesis I presented our expression results of GHRH, pGHRHR, its splicing variants, PTEN and EGFR genes in human glioblastoma samples. Our study provides the first evidence that GHRH and SV1 are present in a substantial part of human GB tissues. We pointed out that expression pattern of GHRH and its SV1 receptors could predict prognosis. Namely GHRH positive, SV1 negative cases showed better overall survival. These results showed that we have moved towards finding the GHRH pathway suitable as a molecular target and a useful prognostic factor in human glioblastomas.

Keywords

neuro-oncology, micro-neurosurgery, mass spectrometry, real time in situ tissue analysis, splicing variant receptors, chemotherapy, radiotherapy

List of the publications related to the thesis verified by the University and National Library

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Doctoral School: Doctoral School of Neurosciences

List of publications related to the dissertation

1. **Mezey, G.**, Treszl, A., Schally, A.V., Block, N.L., Vízkeleti, L., Juhász, A., Klekner, Á., Nagy, J., Balázs, M., Halmos, G., Bognár, L.: Prognosis in human glioblastoma based on expression of ligand growth hormone-releasing hormone, pituitary-type growth hormone-releasing hormone receptor, its splicing variant receptors, EGF receptor and PTEN genes.
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H- 4032 Debrecen, Egyetem tér 1. ¤ E-mail publikaciok@lib.unideb.hu

List of other publications

4. Nagy G., Kemény A.A., Major O., Erőss L., Várady P., **Mezey G.**, Fedorcsák I., Bognár L.: Az intracerebrális cavernomák sugársebészete:Hol tart ma a világ?
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   IF:2.294

Total IF of journals (all publications): 14,666
Total IF of journals (publications related to the dissertation): 10,936

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

17 November, 2014

List of lectures, posters and citable abstracts related to the thesis

J Balog, T Szaniszló, **G Mezey**, L Bognár, Z Takáts Real-time identification of brain cancers in the neurosurical theatre. The association of Mass Spectrometry Applications to the Clinical Lab Conference**,** San Diego **2013.** lecture

G Mezey, L Dobai, L Bognar First year outcome of patients with vestibular schwannoma after rotating gamma system treatment. International Stereotactic Radiosurgery Society Conference, Paris, **2011**. poster

G Mezey Complex neurosurgical and neuro-oncological treatment of intracranial metastases, role of oncoteam. Congress of Hungarian Society of Neurosurgery, Szeged, **2011.** lecture

B Rózsa, A Juhász, B Dezső, G Tóth, T Flaskó, Á Klekner, **G Mezey**, L Bognár, A Molnár, C Kiss, A Gyetvai Gene expression of gonadotropin hormone-releasing hormone-I (GnRH-I), gonadotropin hormone-releasing hormone receptor-I (GnRHR-I) and its splicing variant receptors in human benign and malignant tissues, Hungarian Cancer Society XXVIII. Congress, Budapest, **2009** lecture

G Mezey, J Dobai, I Fedorcsák, L Bognár Gamma-radiosurgical treatment of large-sized cystic metastasis after stereotactic volume reduction., Congress of Hungarian Society of Neurosurgery **2009.** poster

G Mezey, J Tóth, L Novák, L Bognár Primary diffusely disseminated pilocytic astrocytoma: case report and review of the literature. Congress of Hungarian Society of Neuro-oncology **2009**. lecture

Low-grade gliomas, Experiences in Debrecen, case reports I-II. **G Mezey** Congress of Hungarian Society of Neuro-oncology **2009**. lectures

G Csiky, J Dobai, **G Mezey**, I Fedorcsák, L Bognár Treatment of vestibular schwannomas by rotating gamma-radiosurgical system: retrospective study. Congress of Hungarian Society of Neuro-oncology **2009.** poster

G Mezey, L Vízkeleti, S Ecsedi, V Koroknai, T Kiss, I Fedorcsák, A Klekner, M Balázs, L Bognár Alterations of EGFR and PTEN genes in different grade astrocytomas. Congress of Hungarian Society of Neurosurgery **2009.** lecture

P Gergely, **G Mezey**, L Őrfi, I Ember In vivo effect of assorted chemopreventive molecules on DMBA-induced onco-suppresor gene expression in CBA/CA mice. International Conference of Anticancer Research, Kos, Greece, **2008.** poster