Metabolic aggressiveness in benign meningiomas with chromosomal instabilities

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Metabolic aggressiveness in meningioma subgroups

Running title: Metabolic aggressiveness in meningioma subgroups

Précis

Measuring the metabolic phenotype of meningioma intact biopsies at the same time as histopathological analysis may allow early identification of clinically aggressive disease among histologically benign tumors.

Keywords: meningioma; chromosomal instabilities; metabolic aggressiveness; FISH and cytogenetics; NMR metabolic profiling

Abbreviations: HR-MAS, high resolution magic angle spinning; TOCSY, $^{1}\text{H}-^{1}\text{H}$ total correlation spectroscopy; HSQC, heteronuclear single quantum coherence spectroscopy
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Abstract

Meningiomas are often considered benign tumors curable by surgery, but most recurrent meningiomas correspond to histological benign tumors. Since alterations in chromosome 14 among others have suggested clinical aggressiveness and recurrence, determining both the molecular phenotype and the genetic profile may help distinguish tumors with aggressive metabolism. The aim of this study was to achieve higher specificity in the detection of meningioma subgroups by measuring chromosomal instabilities by FISH and cytogenetics and metabolic phenotypes by HR-MAS spectroscopy. We studied 46 meningiomas biopsies with these methodologies. Of these, 34 were of WHO grade I and 12 of WHO grade II. Genetic analysis showed a subgroup of histological benign meningioma with chromosomal instabilities. The metabolic phenotype of this subgroup indicated an aggressive metabolism resembling that observed for atypical meningioma. According to the metabolic profiles, these tumors had increased energy demand, higher hypoxic conditions, increased membrane turnover and cell proliferation and possibly increased resistance to apoptosis. Taken together, our results identify distinct metabolic phenotypes for otherwise benign meningiomas based on cytogenetic studies and global metabolic profiles of intact tumors. Measuring the metabolic phenotype of meningioma intact biopsies at the same time as histopathological analysis may allow the early detection of clinically aggressive tumors.
Introduction

Meningiomas are often considered benign tumours curable by surgery. However, around 20% of meningiomas with histological low grade may be clinically aggressive and recur (1). Meningiomas are neoplasms that arise from the leptomeningeal covering of the brain and spinal cord, accounting for 15%–20% of all central nervous system tumours. The current standards for diagnosis of meningiomas are clinical and pathological findings. The World Health Organization (WHO) classifies meningiomas into three histological grades: grade I (benign), grade II (atypical), and grade III (anaplastic) in accordance with the clinical prognosis (2). Signs of malignancy in meningioma include marked vascularity, loss of organoid structure, mitotic figures, nuclear pleomorphism, prominent nucleoli, focal necrosis, or infiltration to the adjacent brain. Atypical and anaplastic meningiomas show a high index of recurrence 5 years after complete resection and are associated with lower survival rates compared to benign meningiomas (3). However, in absolute numbers, most recurrent meningiomas correspond to histological benign tumours. The potential aggressiveness of an individual meningioma is still difficult to evaluate.

The genesis of meningiomas has been associated with loss of genetic material on chromosome 22. Monosomy of this chromosome is the most common genetic alteration in meningioma and was one of the first cytogenetic alterations described in solid tumours (4-5). Loss of 1p and alterations in chromosome 14 are present in many atypical meningioma (6). Losses in 6q, 10 and 18q and gains on 1q, 9q, 12q, 15q, 17q and 20q are also common in atypical meningioma (7). Based on this information, genetic characterization of meningiomas has some value in the sub classification of
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Recent studies show that benign tumours with alterations in chromosome 14 among others may be clinically aggressive and recur (8). However, correlations between a genetic profile and a clinical phenotype, which typically takes time to develop, are affected by many different variables. On the other hand, little is known about the impact of these chromosomal instabilities on the metabolic phenotype of the tumour. The determination of the molecular phenotype simultaneously to the genetic profile may help in further distinguishing metabolically aggressive tumours. This distinction may also aid in determining the aggressiveness of surgical resection and the necessity of combined radiation therapy. Additional criteria for better classification of meningiomas will improve these clinical decisions as well as patient follow up strategy after surgery.

Metabolomics is defined as “the quantitative measurement of the multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification” (9). High resolution NMR spectroscopy of biofluids and tissues combined with multivariate analysis methodologies, like Principal Component Analysis, represent a powerful technique for investigating the metabolome in the area of drug toxicology and disease diagnosis and prognosis (10). NMR is one of the most efficient, robust, reproducible and cheap methods for obtaining metabolic profiles in biological specimens without extensive sample preparation (11). High resolution magic angle spinning NMR spectroscopy (HR-MAS NMR) is a powerful technique for the investigation of metabolites within different intact tissues (12-16). The potential of HR-MAS applications to the study of biological tissues has been widely demonstrated in the investigation of different cellular alterations. In addition, HR-MAS NMR spectroscopy
of intact tissues (ex vivo) provides further advantages over traditional high resolution liquid NMR of tissue extracts (in vitro). This technology can supplement histopathological examination and potentially improve brain tumour diagnostics. HR-MAS spectra generate metabolic profiles that contain information on physiological and pathological status. This approach can be used to define the metabolomic phenotype of a tissue.

The aim of this study was to detect new subgroups of meningioma and to achieve higher specificity in the classification of meningioma by measuring metabolic phenotypes with high resolution NMR spectroscopy. We collected HR-MAS spectra on 34 benign and 12 atypical meningioma tissue samples and used multivariate analysis for detecting metabolic subgroups. Histopathology post-NMR, which is only possible when using HR-MAS methodologies, provides an essential validation of our results. This analysis shows that genetic instability has high impact on the metabolic profile of meningioma samples.
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Materials and methods

Patients and clinical samples

Forty-six human meningiomas biopsies were obtained from 46 patients at the Department of Neurosurgery of the Clinical University Hospital of Valencia. This study was reviewed and approved by the local ethics committee. During surgery, most of the resected tissue was sent for routine histological analysis, and the remainder was immediately put in cryogenic vials and snap-frozen in liquid nitrogen. All snap-frozen samples were stored in a freezer at -80°C until further analysis. All samples used for histopathological examination were fixed in neutral-buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin-eosin. Tumors were classified according to the 2007 WHO Histological Classification (2). Meningioma types analyzed include 34 benign meningiomas (grade I) and 12 atypical meningiomas (grade II).

Cytogenetic analysis and Fluorescence In Situ Hybridization.

Cytogenetic analyses were performed by short-term culture of the tumors. Fresh tumor samples were disaggregated with 2mg/mL of collagenase II. The cells were seeded in flasks using RPMI-1640 medium supplemented with 20% fetal bovine serum, L-glutamine, and antibiotics. The cells were processed after 72 h of culture by a standard technique. Air dried slides were banded by trypsin-Giemsa. Karyotypic analyses were performed according to ISCN (17).

The samples of meningioma used for FISH analysis were studied by tissue microarrays (TMA). We removed four 0.6-mm cores from the corresponding areas on the paraffin block in each case, using the Beecher Instruments Manual Tissue ArrayerI (Beecher...
Instruments, Sun Prairie, WI, USA). For the investigation of chromosome abnormalities by iFISH the probes LSI 22q12, LSI 1p36/LSI 1q25 and LSI t(11;14) IGH/CCND1 (Vysis Inc; Downers Grove, IL) were used.

Hybridizations were performed according to the instructions that accompany the probe. Counterstaining of nuclei was carried out using 4',6-diamidino-2-phenylindole (DAPI). The fluorescent signal was detected using a photomicroscope Axioplan 2 and Axiophot 2 (Zeiss) equipped with a set of the appropriate filters. For each hybridization, green and orange signals were counted in the four regions of a total of 100-200 non-overlapping nuclei. An interpretation of deletion or imbalance was made when >20% of the nuclei harbored these alterations (18-19). Cutoffs for deletions were based on the frequencies of signals for the same probes in non-neoplastic brain controls (median ±3) and ranged from 14 to 21% for chromosome 1, from 16 to 22% for chromosome 14, and from 15 to 20% for monosomy 22. We considered deletion when appeared one or less signal for chromosome with respect to the signal of control (ratio 0/1, 0/2, 1/2, 1/3…) and we considered normal with the probes used present a ratio 2/2.

**HR-MAS spectroscopy**

Total sample preparation time for each sample prior to NMR detection was less than 5 min. All the material to be in contact with the tissue was pre-cooled to reduce tissue degradation during the sample preparation process. Frozen samples were taken from the ultra-freezer and immediately placed in a cryo-vial and in liquid N₂ until insertion in a 4-mm outer diameter ZrO₂ rotor. The HR-MAS tissue sample was split from the whole frozen tumoral mass submerged in liquid nitrogen. The pre-cooled rotor was filled with
cooled D$_2$O after tissue sample insertion. Cylindrical inserts were used in all the cases, limiting the rotor inner volume to 50 μl. Exceeding D$_2$O was removed before rotor sealing. Tissue samples were weighted in the rotor before D$_2$O addition and HR-MAS measurements. Tissue fragments were weighted exclusively for sample preparation purposes. The mean sample weight was 27 ± 11 mg. Immediately after the measurement, the HR-MAS samples were fixed in formalin for subsequent histopathological examination and for tumor content assessment by an expert pathologist.

All spectra were recorded in a Bruker Avance DRX 600 spectrometer (Valencia, Spain) operating at a $^1$H frequency of 600.13 MHz. The instrument was equipped with a 4 mm triple resonance $^1$H/$^{13}$C/$^{15}$N HR-MAS probe with magnetic field gradients aligned with the magic angle axis. For all experiments, samples were spun at 5000 Hz to keep the rotation sidebands out of the acquisition window. Lock homogeneity was achieved by extensive coil-shimming using the 1D water pre-saturation experiments in interactive mode as control. Alanine doublet at 1.475 ppm was used for lock homogeneity shimming, as described elsewhere (20). Nominal temperature of the sample receptacle was kept at 273K, using the cooling of the inlet gas pressures responsible for the sample spinning. This value corresponded to the temperature measured from the thermocouple just below the rotor in the probe. The effect of sample rotation was to slightly increase this value. Internal measurement using a 100% MeOH sample in a 4 mm rotor spinning at the same frequency provided a corrected internal value of 277K. In order to minimize the effects of tissue degradation, which would alter the metabolite composition of the biopsy, all ex vivo spectra were acquired at this temperature of 277K. A total of 10 min
was allowed for the temperature of the sample to reach steady state before spectra were acquired. A single-pulse pre-saturation experiment was acquired in all the samples. Number of transients was 256 collected into 32 k data points for all the experiments. Water pre-saturation was used during 1 sec along the recycling delay for solvent signal suppression. Spectral widths were 8000 Hz for 1H. Before Fourier transformation, the free induction decay was multiplied with a 0.3 Hz exponential line broadening. Chemical shift referencing was performed relative to the Alanine CH$_3$ signal at 1.475 ppm. For assignment purposes, two-dimensional (2D) homo (2D-TOCSY) and heteronuclear (2D-1H, 13C-HSQC) experiments were acquired on selected samples.

**NMR data analysis**

All 30 spectra were processed using MNova 5.3 (MestreLab S.A., Santiago de Compostela, Spain) and transferred to MATLAB (MathWorks Inc, 2006) using in-house scripts for data analysis. All multivariate analysis was performed using the PLS_Toolbox library. The chemical shift region including resonances between 0.50 and 4.60 ppm and between 5.20 and 10.50 was investigated. For comparison of global metabolic profiles from different meningioma subgroups, the spectra were binned into 0.01 ppm buckets, normalized to total spectral integral and subsequently analyzed by Principal Components Analysis (PCA). The spectral binning and normalization minimized the impact of differences in tissue weight and cell content for the different biopsies. We cross validated our PCA model by performing 10 technical replicates by choosing random training (36 samples) and validation (10 samples) data subsets. Cross-validation is a technique for assessing how the results of a statistical analysis will generalize to an independent data set. The first two principal components of the average
model (PC1 and PC2) accounted for a total of 65% of the variance in the spectral data set. Lower order Principal Components did not provide clear differences between subgroups and consequently were not further analyzed. Spectral signal integration by peak-fitting algorithms over relevant resonances provided relative levels of the corresponding metabolites. Only those signals with peak-fitting residual error lower than 10% were used in the study.

Statistical analysis

To determine individual metabolic differences between the different subgroups of meningioma univariate t-student tests were performed for each signal of the individual metabolites detected as major contributor in the PCA analysis. We calculated the maximum false discovery rate (FDR) of the metabolites selected to account for multiple testing. FDR was estimated using the q-value statistical parameter (21). The q-value gives the estimated FDR for every possible list of significant signals. The q-value is based on estimating the true proportion of null hypotheses. The p-value histogram width used for calculating q-values was 0.005. Since we performed three comparisons and the q-value may be different, the q-value was applied separately to the p-values of each comparison. Unless otherwise indicated, the limit of q-value for metabolites selected in each comparison was set to 0.05 (FDR of 5%).

Results

Histopathology

Histological analysis of the tissue specimens after HR-MAS analysis showed various amounts of tumor tissue (Figure 1). In all cases histological analysis of the HR-MAS
sample agreed with the histopathology original diagnosis. All samples included a percentage of tumour cells at least of 80%, as evaluated by two expert pathologist exploring at 10x the whole sample (1 to 2 mm), confirming that samples are representative of the tumour, independently of tumour grade.

Chromosomal profile

Karyotyping was obtained in 37 cell cultures from 37 different cases. Sample material was adequate for FISH studies in 42 cases. Information from both methodologies was combined to distinguish complex chromosomal profiles. Thirty seven out of 46 meningiomas presented clonal numerical and/or structural abnormalities, as shown in Table 1 and Supplementary Material. All cases without chromosomal aberrations belonged to the histological grade I meningioma group. Monosomy of chromosome 22 is the most common genetic alteration in meningioma and was detected in 22 of all cases. All cases without a complex karyotype were of histological grade I. Similarly, all those cases with alterations in chromosome 22 as the only chromosomal anomaly were also of histological grade I. According to FISH chromosomal analysis, only 16% (5 out of 32) of benign meningiomas are -1p whereas this percentage rises to 70% (7 out of 10) in atypical meningioma. All cases containing alterations in chromosome 14 were of histological grade II. All grade II meningiomas showed a complex karyotype with 6 of them showing chromosomal alterations different to -1p, -22 and/or -14. These findings confirm that exploration of chromosomes beyond 1, 14 and 22 is recommended for genetic characterization of meningiomas. Our results show that meningiomas with higher histological grade (grade II) are very likely to have a complex karyotype. There is a subpopulation of meningiomas with histological grade I which also exhibits
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complex karyotype, despite not having alterations in chromosome 14. Besides 1, 14 and 22, chromosomes affected either in this benign meningioma subgroup or in atypical meningioma include 2, 3, 5, 7, 9, 10, 17 and 18. Differently to some previous studies, the combined use of FISH and cytogenetics allowed the detection of many different anomalous chromosomal profiles. Based on chromosomal profiles, we defined three meningioma subgroups for subsequent analysis: histological grade I with normal or diploid karyotype (benignA), histological grade I with complex karyotype (benignB) and histological grade II (atypical).

Metabolic profile

HR-MAS spectroscopy provided well resolved spectra of intact meningioma tissue samples. NMR spectra of benignA, benignB and atypical meningioma are displayed in Figure 1. The PCA analysis (Figure 2) showed partial group separation between meningiomas benignA and meningiomas with complex karyotype (benignB and atypical). On the other hand, histological benign and atypical meningiomas formed two partially overlapping groups. The overlapping region contained most of the meningioma tumours showing chromosomal instabilities, indicating metabolic similarity between benign meningiomas with chromosomal instabilities (benignB subgroup) and the more aggressive atypical meningioma tumours. In the next step, individual metabolites were identified in the PCA loadings plot and quantified for further analysis. A total of 19 metabolites showed statistically significant differences between either two of the three subgroups (Table 1). No statistically significant correlation was detected between FISH quantitative data (see Supplementary Material) and the metabolic profiles.
The major features for separation between benign meningioma and atypical meningioma, the two subgroups clearly separated in the PCA scores plot, were higher concentrations of glycine, glutamate, total glutathione, lactate, taurine, phosphocholine, phosphoethanolamine and uracil and lower concentrations of global fatty acids, γ-aminobutyric acid, ascorbate, acetate, creatine, alanine, leucine, isoleucine, glutamine, ascorbate, lysine, glucose, choline and ethanolamine. For most of these metabolites, with the exception of taurine, glutamine and γ-aminobutyric acid, the values for benign meningioma were closer to atypical meningioma than to benign meningioma (Table 1 and Figure 3). Meningioma with complex karyotype showed increased levels of glutamate and total glutathione and reduced levels of glutamine, suggesting an increase in total glutathione production. Glucose, ascorbate, acetate and fatty acids, the four of them metabolites involved in the TCA cycle and fatty acids β-oxidation were all decreased in the same groups. Conversely, lactate was higher in atypical meningioma. Increased glucose uptake and high glycolytic activity, due to high energy requirement, are major hallmarks of tumour metabolism. These changes usually result in decreased intracellular glucose concentrations and higher levels of lactate. However, these metabolites may also be altered by the biopsy extraction procedure. Phosphocholine and phosphoethanolamine, both phospholipids derivatives, also showed increased levels in meningioma groups with complex karyotype. Increased membrane metabolites turnover is another significant metabolic pathway for tumour development and aggressiveness. On the other hand, the similar levels of polyunsaturated and monounsaturated fatty acids in all groups suggested a similar amount of necrotic fraction in the tissue. Finally, despite high variability, uracil metabolites increased with complex karyotype progressively and in a statistically significant manner. This suggests that nucleotide
metabolism is also altered in more aggressive tumours.

Discussion

Most meningiomas are classified as benign based on clinical and pathological findings. Nonetheless, the behavior of individual meningioma is still difficult to predict. Many previous studies report some correlation between chromosomal alterations and meningioma clinical outcome. Typically, these correlations are usually weak because of two reasons. First, the chromosomal profile typically is limited to FISH analysis of a few chromosomes. Second, clinical outcome takes years to be properly assessed and still many individuals may exhibit the clinical aggressiveness later on. Maillo et al show that statistical correlation between genetic abnormalities and clinical outcome improves when follow up is extended to 10 years, with a subset of patients showing recurrence 15 years after surgery (22). On the other hand, most of the previously reported meningioma subgroups define aggressiveness based solely on WHO grade and morphological parameters. This definition of the phenotype of an aggressive meningioma is rather poor. The metabolic changes of any cell population precede morphological changes. In fact, chromosomal alterations produce changes in the metabolic phenotype almost immediately. The measurement of a metabolic phenotype at the moment of the histopathological diagnosis may help in detecting better defined molecular meningioma subgroups.

In our study, chromosomal profiling by cytogenetics and FISH and metabolic profiling by NMR spectroscopy on benign and atypical meningioma reveal distinct molecular phenotypes that allowed detecting metabolic aggressiveness in these tumours. Previous
studies on metabolic profiling of meningiomas report weak correlations between grade and metabolic phenotype (19, 23). This may be due to the use of tissue extracts which precludes the histopathology of the sample and makes impossible to know the tumor content, leading to much broader variability in the metabolite levels. To our knowledge, this is the first combined genetic and metabolic analysis of intact biopsies of benign and atypical meningiomas for detecting metabolic subgroups. The use of cytogenetics allows the detection of a global chromosomal profile. Similarly, NMR spectroscopy combined with multivariate analysis provides a global metabolic profile. The combination of these data provides a robust and well delimited set of meningioma subgroups.

Chromosomal aberrations in a tumour sample are typically studied by FISH. FISH is a target-oriented method that allows direct observation of specific chromosomal abnormalities. It is more sensitive than standard cytogenetics methods because it does not require dividing cells and can be measured directly in the tumour tissue. However, cytogenetics methods provide a whole genome chromosomal profiling and may reveal chromosomal alterations in regions different to those explored by target oriented methods. The combination of both methods provides a global chromosomal profile with high sensitivity in specific regions. Therefore, we found chromosomal alterations that would not have been detected by any of the individual approaches alone. We chose to examine chromosomes 1, 14 and 22 by FISH because aberrations of those chromosomes are the most frequently reported genetic abnormalities in meningioma (7). Our findings confirmed that loss of 1p and/or 14 are the most common chromosomal alterations in atypical meningioma. Coexistence of monosomy 14 and 1p is a powerful adverse
prognostic factor for early relapses (6). However, meningiomas are cytogenetically heterogeneous tumours. The detection of a subset of benign meningiomas with chromosomal alterations beyond monosomy 22 may have an important impact in defining aggressiveness in meningiomas. This subset of benign meningiomas (benignB subgroup) shows a wide variety of chromosomal alterations all producing a complex karyotype. Histological benign meningiomas with complex karyotype have higher probability of relapsing (22). In line with this, in the present study, all histological benign meningiomas with complex karyotype (subgroup benignB) demonstrate a metabolic phenotype similar to that of atypical meningioma.

The metabolic phenotype of meningiomas with complex karyotype shows typical features of aggressive tumor biochemistry like increased membrane metabolites turnover and high glycolytic activity. Lower levels of glucose and ascorbate together to higher levels of lactate suggest higher dependence on anaerobic breakdown of pyruvate for meningiomas with complex karyotype. Lactate accumulation also creates a microenvironment which may enhance cell proliferation (24). Lower glucose levels in cells of more aggressive tumours may reflect preferential glucose consumption and a local hypoxic microenvironment. Hypoxia in the tumor microenvironment triggers a variety of genetic and adaptive responses that regulate tumor growth (25). Tumor hypoxia is often associated with more malignant phenotypes, resistance to therapy, and poor survival. Another metabolite closely related to energy metabolism is creatine. Total creatine is lower in meningiomas with chromosomal instabilities. This may indicate either lower substrate availability or increased ATP usage, which suggests an environment with high energy demand. Finally, the decreased levels of fatty acids may
also reflect increased β-oxidation. All these processes result in a metabolically unfavorable microenvironment with low glucose availability and local hypoxic conditions. Our results suggest that meningiomas with chromosomal instabilities have this energy demanding microenvironment.

Choline-containing compounds are elevated in tumors (26-27). Phosphocholine and choline compounds are generally considered as well established proliferation markers. In addition, high phosphocholine is also correlated with highly invasive phenotypes. However, these compounds are not easily detected by conventional high resolution NMR. The tissue extraction process disables the direct observation of small cellular proteins and membrane semi-mobile phospholipids precursors. On the contrary, HR-MAS allows the determination of these important metabolites in intact biopsies. Meningiomas with chromosomal instabilities have higher phosphocholine levels, which are consistent with higher proliferative rates. In addition, phosphoethanolamine, which is also considered as an intermediate in the metabolism of phospholipids, exhibits increased levels in meningioma with chromosomal instabilities. A high relative intensity of the phosphoethanolamine resonance in 31P MR spectroscopy is a reported marker for malignancy (28). Increased phosphoethanolamine levels suggest an activation of phosphatidylethanolamine metabolism, which is involved in the modification of membrane shape in malignant tissue (29). Additionally, free phosphoethanolamine might reflect the breakdown of phosphatydilethanolamine to diacylglycerol, and might act as a long-term second-messenger for cell proliferation (30).

Glutathione is an important cell antioxidant and, in normal tissues, plays different roles
in protection against oxidative damage (31), in apoptosis (32-34) and in amino acid transport (35). Glutathione depletion is a common feature of apoptotic cell death. In cancer, glutathione is able to play both protective and pathogenic roles (31). In addition, there is a correlation between decreased total glutathione levels and cell differentiation. In meningioma, levels of glutathione and closely related metabolites, like glutamate, are increased with respect other brain tumors and healthy brain tissue (36). A potential explanation for it is the reversible transamination reaction of alanine and α-ketoglutarate, synthetising glutamate, as an alternative energy source. This may also explain the lower levels of alanine in atypical meningioma despite higher hypoxic conditions. Another source of glutamate is glutamine by the glutaminase reaction, which also explains the lower levels found for glutamine in the same subgroup. Meningioma with chromosomal instabilities shows increased levels of glutathione and glutamate and decrease levels of alanine and glutamine, which suggest less cell differentiation, resistance to apoptosis and activation of alternative energy sources.

The aforementioned metabolites and pathways play critical roles in cancer metabolism. All of them suggest that meningiomas with chromosomal instabilities suffer metabolic alterations related to aggressiveness, proliferation and invasiveness (see Figure 4 for a schematic view). Other metabolic changes reported here are also in the same line. For example, glycine, which is the simplest non-essential amino acid and is associated to recurrence in some brain tumours (37), is increased in meningioma with complex karyotype. Glycine is a major component of collagen. Increased levels of glycine suggest increased collagen synthesis and artery walls formation, and therefore sustained angiogenesis. Increased levels of uracil in meningioma with chromosomal instabilities
may reflect the activation of uracil glycosylase, which excises uracil from DNA deaminated cytosine and repairs damaged DNA. Previous studies reported that uracil glycosylase activity correlates with tumour recurrence (38).

In summary, the metabolic phenotype detected by HR-MAS in meningioma allows detecting metabolic aggressiveness in histological benign tumours. The multivariate analysis shows that benign meningioma with complex karyotype (benignB subgroup) are metabolically closer to atypical meningioma than to other benign meningiomas (benignA subgroup). Most of the metabolites showing statistically significant differences between groups suggest a more aggressive biochemistry in meningiomas with chromosomal instabilities regardless of their histological grade. To our knowledge, this is the first time that distinct metabolic phenotypes are reported for otherwise benign meningiomas based on cytogenetic studies and multivariate analysis and global metabolic profiling of intact tumour biopsies. Overall, this work shows that the measurement of a metabolic phenotype in meningioma intact biopsies simultaneously to the histopathology analysis may allow the early detection of metabolically aggressive tumors.

Acknowledgements

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Table 1. Summary of chromosomal instabilities detected in benign and atypical meningioma according to cytogenetic and FISH analysis. Cells in gray show the chromosomal instabilities progression in the different subgroups of meningioma.

<table>
<thead>
<tr>
<th></th>
<th>Chromosomal instabilities</th>
<th>FISH</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal -22 -1p -14 Others* Non-clonal** -22q -1p -14q Diploid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BenignA</td>
<td>8 7 - - - - 10 - - 8 20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BenignB</td>
<td>- 9 3 13 5 9 5 - 3 14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atypical</td>
<td>- 6 3 4 7 2 7 7 4 - 12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Numeric or structural alterations different to Monosomy 22, 1p deletion and Monosomy 14 (see supplementary material)
** Numeric or structural alterations which are present only once in the different metaphases analyzed
Table 2. Resonance intensity ratios, standard deviations and p-values from most relevant metabolites in the classification of benign and atypical meningiomas. Most of the metabolites show values for BenignB meningioma closer to Atypical than to BenignA meningioma suggesting metabolic aggressiveness in this subgroup.

<table>
<thead>
<tr>
<th>Metabolite (peak position)</th>
<th>BenignA (Relative intensity (a.u.))</th>
<th>BenignB (Relative intensity (a.u.))</th>
<th>Atypical (Relative intensity (a.u.))</th>
<th>p-value BenignA vs BenignB</th>
<th>p-value BenignA vs Atypical</th>
<th>p-value BenignB vs Atypical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acids (0.89 ppm)</td>
<td>73±24</td>
<td>45±18</td>
<td>52±21</td>
<td>0.008</td>
<td>0.016</td>
<td>0.388</td>
</tr>
<tr>
<td>Leucine (0.97 ppm)</td>
<td>45±15</td>
<td>30±10</td>
<td>34±12</td>
<td>0.019</td>
<td>0.072</td>
<td>0.333</td>
</tr>
<tr>
<td>Isoleucine (1.02 ppm)</td>
<td>28±7</td>
<td>20±6</td>
<td>23±7</td>
<td>0.008</td>
<td>0.011</td>
<td>0.141</td>
</tr>
<tr>
<td>Lactate (1.33 ppm)</td>
<td>211±121</td>
<td>302±99</td>
<td>341±132</td>
<td>0.028</td>
<td>0.008</td>
<td>0.401</td>
</tr>
<tr>
<td>Alanine (1.48 ppm)</td>
<td>58±24</td>
<td>29±18</td>
<td>31±17</td>
<td>0.001</td>
<td>0.002</td>
<td>0.797</td>
</tr>
<tr>
<td>Acetate (1.92 ppm)</td>
<td>21±8</td>
<td>16±8</td>
<td>15±7</td>
<td>0.078</td>
<td>0.032</td>
<td>0.662</td>
</tr>
<tr>
<td>Glutamate (2.35 ppm)</td>
<td>23±4</td>
<td>27±7</td>
<td>31±8</td>
<td>0.014</td>
<td>0.265</td>
<td>0.037</td>
</tr>
<tr>
<td>Glutamine (2.44 ppm)</td>
<td>30±9</td>
<td>30±18</td>
<td>23±5</td>
<td>0.978</td>
<td>0.059</td>
<td>0.256</td>
</tr>
<tr>
<td>Total glutathione (2.55 ppm)</td>
<td>12±6</td>
<td>17±4</td>
<td>17±8</td>
<td>0.081</td>
<td>0.009</td>
<td>0.913</td>
</tr>
<tr>
<td>Creatine (3.03 ppm)</td>
<td>48±22</td>
<td>29±17</td>
<td>25±13</td>
<td>0.011</td>
<td>0.004</td>
<td>0.595</td>
</tr>
<tr>
<td>Ethanolamine (3.15 ppm)</td>
<td>10±3</td>
<td>8±3</td>
<td>7±3</td>
<td>0.051</td>
<td>0.012</td>
<td>0.150</td>
</tr>
<tr>
<td>Choline (3.19 ppm)</td>
<td>43±12</td>
<td>30±12</td>
<td>29±15</td>
<td>0.012</td>
<td>0.011</td>
<td>0.731</td>
</tr>
<tr>
<td>Phosphocholine (3.22 ppm)</td>
<td>61±35</td>
<td>82±28</td>
<td>91±40</td>
<td>0.077</td>
<td>0.043</td>
<td>0.551</td>
</tr>
<tr>
<td>Taurine (3.26 ppm)</td>
<td>25±8</td>
<td>26±8</td>
<td>32±9</td>
<td>0.745</td>
<td>0.025</td>
<td>0.064</td>
</tr>
<tr>
<td>Glycine (3.55 ppm)</td>
<td>26±8</td>
<td>30±7</td>
<td>34±9</td>
<td>0.361</td>
<td>0.030</td>
<td>0.167</td>
</tr>
<tr>
<td>Glucose (3.88 ppm)</td>
<td>41±17</td>
<td>36±14</td>
<td>28±11</td>
<td>0.317</td>
<td>0.011</td>
<td>0.095</td>
</tr>
<tr>
<td>Phosphoethanolamine (4.01 ppm)</td>
<td>14±7</td>
<td>15±6</td>
<td>23±9</td>
<td>0.839</td>
<td>0.008</td>
<td>0.008</td>
</tr>
<tr>
<td>Ascorbate (4.52 ppm)</td>
<td>2±1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Uracil (7.54 ppm) **</td>
<td>-</td>
<td>2±1</td>
<td>2±1</td>
<td>-</td>
<td>-</td>
<td>0.983</td>
</tr>
</tbody>
</table>

* q-value greater than 0.05
** Signal intensity was not sufficient for quantification in all the samples
Figure legends

Figure 1. Representative NMR spectra (panels A, B, C), FISH images (panels D, E and F) and histopathology (panels G, H and I) for the benign meningioma without chromosomal instabilities (meningothelial meningioma, subgroup benignA, panels A, D and G), benign meningioma with chromosomal instabilities (transitional meningioma, subgroup benignB, panels B, E and H) and atypical meningioma (panels C, F and I). Resonances belonging to metabolites with statistical significance have been labeled in the spectra (1: Fatty acids, 2: Leucine, 3: Isoleucine, 4: Lactate, 5: Alanine, 6: Acetate, 7: Glutamate, 8: Glutamine, 9: Total glutathione, 10: Creatine, 11: Ethanolamine, 12: Choline, 13: Phosphocholine, 14: Taurine, 15: Glycine, 16: Glucose, 17: Phosphoethanolamine). FISH images show chromosomes 22 (panel D, normal), 1 (panel E, 1p deletion as red spots) and 14 (panel F, 14q deletion as green spots).

Figure 2. Scores plot of Principal Component Analysis to compare the metabonome of the benign meningioma without chromosomal instabilities (subgroup benignA, white circles), the benign meningioma with chromosomal instabilities (subgroup benignB, gray circles) and the atypical meningioma (black circles).

Figure 3. Histograms showing relevant metabolite levels, standard deviation error bars and significance levels (1 star means p value less than 0.05, 2 stars mean p value less than 0.02 and 3 stars mean p value less than 0.01) for the benign meningioma without chromosomal instabilities (subgroup benignA, balck bars), the benign meningioma with chromosomal instabilities (subgroup benignB, gray bars) and the atypical meningioma (white bars). Label code: FA, fatty acids; Glc, glucose; Ace, acetate; Lac, lactate; Ala,
alanine; Cre, creatine; Gsx, Total glutathione; Tau, taurine; Cho, choline; EA, ethanolamine; PCho, phosphocholine; PEA, phosphoethanolamine.

Figure 4. Scheme showing the interconnection between the different metabolites and processes involved in the metabolic aggressiveness observed for the benign meningioma with chromosomal instabilities. Essentially the metabolic differences observed in benign meningioma with chromosomal instabilities with respect other benign meningioma suggest higher energy demand and metabolic activity, higher proliferation rates, higher antioxidant capacity and higher hypoxia, which are indicative of more aggressive tumors. Label code: FA, fatty acids; Glc, glucose; Ace, acetate; Lac, lactate; Ala, alanine; Cre, creatine; Gsx, Total glutathione; Glx, glutamine plus glutamate; Tau, taurine; Cho, choline; EA, ethanolamine; PCho, phosphocholine; PEA, phosphoethanolamine.
Metabolic aggressiveness in benign meningiomas with chromosomal instabilities

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