

# Combining magnetic resonance spectroscopy and molecular genomics offers better accuracy in brain tumor typing and prediction of survival than either methodology alone

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**Abstract.** Recent advents in magnetic resonance spectroscopy (MRS) techniques permit subsequent microarray analysis over the entire human transcriptome in the same tissue biopsies. However, extracting information from such immense quantities of data is limited by difficulties in recognizing and evaluating the relevant patterns of apparent gene expression in the context of the existing knowledge of phenotypes by histopathology. Using a quantitative approach derived from a knowledge base of pathology findings, we present a novel methodology used to process genome-wide transcription and MRS data. This methodology was tested to examine metabolite and genome-wide profiles in MRS and RNA in 55 biopsies from human subjects with brain tumors with ~100% certainty. With the guidance of histopathology and clinical outcome, 15 genes with the assistance of 15 MRS metabolites were able to be distinguished by tumor categories and the prediction of survival was better than when either method was used alone.

This new method, combining MRS, genomics, statistics and biological content, improves the typing and understanding of the complexity of human brain tumors, and assists in the search for novel tumor biomarkers. It is an important step for novel drug development, it generates testable hypotheses regarding neoplasia and promises to guide human brain tumor therapy provided improved *in vivo* methods for monitoring response to therapy are developed.

## Introduction

According to the Central Brain Tumor Registry of the USA ([www.cbtrus.org](http://www.cbtrus.org)), the worldwide incidence rate of primary malignant brain and central nervous system (CNS) tumors, age-adjusted using the world standard population, is 3.7 per 100,000 person-years in males and 2.6 per 100,000 person-years in females. The incidence rates are higher in more developed countries (males, 5.8 per 100,000 person-years; females, 4.1 per 100,000 person-years) than in less developed countries (males, 3.0 per 100,000 person-years; females, 2.1 per 100,000 person-years).

Management of brain tumors in patients would benefit from improved characterization, diagnosis and prognostic biomarkers. The diagnostic utility of biomarkers for tissue typing lies in their biological relevance. Highly informative biomarker profiles are difficult to establish, due to current technical limitations and the small sample sizes of tissue biopsies, which pose challenges for producing accurate magnetic resonance spectroscopy (MRS) and transcriptome data. Further development and application of microscale MRS and genomics can overcome these limitations and identify new biomarkers to accurately type cancers. This report focuses on applying these technologies to brain tumors, the leading cause for high mortality in older adults (1,2).

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Our hypothesis is that current tissue characterization is enhanced by developing and applying a classification strategy analysis algorithm that produces unique tumor fingerprints by combining biomarker profiles from *ex vivo* MRS and whole-genome expression profiling performed on microscale pediatric brain tumor biopsies. Brain-tumor specific biomarkers can be identified using high-resolution *ex vivo* MRS at high magnetic field strengths and a combination of microarray, bioinformatics, and computational analyses. In the near future, combining clinical MRI, MRS and MR imaging of gene expression *in vivo* should produce superior images to enhance the specificity of cancer diagnosis in clinical medicine. Knowledge acquired from these studies can also be used to type inoperable cancers. This hypothesis builds upon prior reports (3-16). Prior data have provided the following information and advances: a) measurements using one-dimensional (1D) *ex vivo* High-Resolution Magic Angle Spinning (HRMAS) proton ( $^1\text{H}$ ) MRS at 9.4 T correlated directly with the neuropathology of intact brain tumor biopsies. The intracellular metabolite phosphocholine (PCho), a constituent of the choline (Cho) peak, was associated with cellularity and proliferative activity (17); b) MRS-detectable lipid changes were implicated in brain tumor apoptosis and necrosis (17); c) *in vivo* MRS spectra correlated with *ex vivo* MRS measurements (17); d) Gene expression analyses of embryonal CNS tumors distinguished between tumor types that could not be clearly distinguished by histopathology (18,19); e) HRMAS  $^1\text{H}$  MRS and genomic analyses of microscale tissue biopsies (~2 mg) yielded quality data, enabling MRS-derived metabolites and gene expression differences to be related and cross validated (15); f) an optimized adiabatic solid-state NMR method, Total Through-Bond Spectroscopy (TOBSY), maximized the advantages of HRMAS applied to intact biopsies when compared to more conventional liquid-state NMR approaches (20,21); g) a structured network knowledge-based approach was demonstrated to be capable of analyzing genome-wide transcriptional responses in the context of known functional interrelationships among proteins, small molecules, and phenotypes (13); and h) the design and initial testing of our classification algorithms was successful (22).

While considerable work has been done on the classification of cancers based on genomic data (23-33), and some work has been done using MRS data (22,34-42), these two datasets have yet to be integrated along with other clinical features. We hope to improve cancer diagnosis accuracy immediately following biopsy collection by uncovering and exploiting complementary information in the MRS and genomic data. We undertook this challenge by employing machine-learning methods that combine the data generated from MRS and genomics. Our rationale for using methods that emerged under the machine-learning framework, such as the support vector machines (SVM), is that these methods have already been employed successfully for cancer classification for more than ten years (25,32,43-45) and have replaced other traditional methods such as linear discriminant analysis, logistic regression, density-based methods (Parzen window, Naïve Bayes), neural networks with PCA pre-processing, and decision trees (32). As a result of its wide acceptance as a state-of-the-art method for gene-based diagnosis, the SVM constitutes an important module of modern software tools for gene expression analysis

(46). On the other hand, statistical methods are limited mostly to the task of ranking individual genes with respect to their ability to act as individual markers, thus they can be useful in the feature selection task. Also, the SVM approach has been proposed for classification of heterogeneous data sources using a weighted combination of multiple similarity measures (kernels), (one kernel for each data source). This methodology has already been successfully applied in several medical diagnosis tasks (47,48) and is a promising technique for combining data from multiple sources. In our line of research, the data sources to be combined are genomics, MRS, and clinical data. Here, we report that a new classification system using the sensor fusion approach, which combines genomics, MRS, statistics and biological content, improves the typing and understanding of the complexity of human brain tumors, as well as the search for novel tumor biomarkers, an important step for novel drug development. It also generates testable hypotheses regarding neoplasia and promises to guide human brain tumor therapy provided that improved *in vivo* methods for monitoring response to therapy are developed.

## Materials and methods

**Experimental design.** We carried out experiments on a dataset of 55 gene expression profiles derived from normal (9 cases) and tumor (46 cases) classes. The tumor class samples belonged to three categories: high grade (H) [20 cases, 12 glioblastoma multiforme (GBM); 8 anaplastic astrocytoma (AA)], low grade (L) (17 cases, 7 meningioma; 7 schwannoma; 7 pilocytic astrocytoma) and metastasized (M) (11 cases, 5 adenocarcinoma; 3 breast cancer metastasis; 3 other metastasis). Subjects ranged in age from 17 to 54 years.

**HRMAS  $^1\text{H}$  MRS using adiabatic TOBSY.** We used a previously designed 2D *ex vivo* HRMAS  $^1\text{H}$  MRS procedure for brain tumors, based on novel concepts rooted in solid-state NMR spectroscopy (49). All HRMAS  $^1\text{H}$  MRS using TOBSY experiments were performed on a Bruker BioSpin Avance NMR spectrometer (600.13 MHz) using a 4-mm triple resonance ( $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^2\text{H}$ ) HRMAS probe (Bruker). Specimens were pre-weighed and transferred to a  $\text{ZrO}_2$  rotor tube (4 mm diameter, 50  $\mu\text{l}$ ), containing an external standard [trimethylsilyl propionic-2,2,3,3-d $_4$  acid (TSP),  $M_w=172$ ,  $d=0.00$  ppm] that functioned as a reference both for resonance chemical shift and quantification. The HRMAS  $^1\text{H}$  MRS was performed at  $-8^\circ\text{C}$  with 3 kHz MAS speed to minimize tissue degradation. Typical acquisition parameters were, 2 k points direct dimension (13 ppm spectral width), 200 points indirect dimension (7.5 ppm spectral width), 8 scans with 2 dummy scans, 1 sec water pre-saturation, 2 sec total repetition time, 45 msec mixing time and total acquisition time 45 min.

**Analysis of 2D TOBSY MR spectra.** The spectra of intact specimens were analyzed using the XWINNMR 3.5 software package (Bruker Biospin Corp, Billerica, MA). Before Fourier transformation and phasing, the 2D free induction decays were subjected to QSINE=3 window apodization. Baseline correction was then performed using a low order spline function. After Lorentzian and/or Gaussian fitting, the area under the curves or the volumes of the 15 most intense spectra resonances were

calculated. Relative quantification using the TSP standard was performed as described below. These resonances were identified and assigned to the corresponding metabolites.

**Quantification of brain metabolites from the 2D TOBSY MR spectra.** To quantify the brain metabolites, we used the ratio of the cross peak volumes of the metabolites [CPV(M)] to the TSP diagonal peak volume [DPV(TSP)]. This ratio was further divided by the biopsy weight (w) to yield the normalized metabolite intensity,  $I_c = 1/w \times \text{CPV(M)}/\text{DPV(TSP)}$ .

**Microscale transcriptome analyses to determine the gene expression profiles of the tumor biopsy samples after HRMAS <sup>1</sup>H MRS.** We performed microscale genome array studies with the commercially available Affymetrix U133Plus<sup>®</sup> array (Santa Clara, CA).

**Platform choice.** The Affymetrix GeneChip<sup>®</sup> DNA microarray platform has several significant advantages over competing technologies, including coverage of the entire human genome, access to probe sequences, probe redundancy (11 sequences per gene) to optimize fidelity of the signal-to-noise ratio, ready commercial availability, standardization of hybridization, washing, staining and scanning processes, quality control built into the manufacturing processes, available technical support, and a relatively low cost per investigated gene.

**RNA purification.** Total experimental RNA were isolated from the biopsy samples used for HRMAS <sup>1</sup>H MRS. Total control RNA was isolated from normal tissue removed along with the tumor biopsies, or from age-matched patients undergoing epilepsy surgery. RNA was isolated using the modified protocol of the RNeasy purification kit (Qiagen) that our Stanford colleagues have optimized. Briefly, during tissue homogenization and deproteination, 1 mg of tRNA and 10 mg linear polyacrylamide are added as carriers. This greatly improves RNA yields to ~500 ng of total RNA per mg of tissue, an amount x20 greater than that required for our optimized RNA labeling procedure. RNA purity was assessed from the OD 260/280 ratio, with only samples having ratios >1.9 retained for further use. In addition, RNA integrity was assessed by the Agilent 2100 Bioanalyzer, where good quality samples exhibit a relatively flat and low baseline in the capillary electrophoresis elution and have 18S and 28S peaks between 1:1 to 1:2, as scored by the Bioanalyzer software.

**RNA labeling.** We used the Ribo-SPIA protocol ([www.nugeninc.com](http://www.nugeninc.com)) for mRNA labeling and amplification. Ribo-SPIA is superior to all other labeling methods, when the amount of RNA is <1 mg. An overview of the Ribo-SPIA amplification process was described previously by Tzika *et al.*, (15). We used 20 ng total RNA for first strand cDNA synthesis, and the entire procedure for amplification, fragmentation and labeling was performed in one day.

**Data analysis of gene expression.** The expression profiles of tumor biopsies analyzed and compared to those of control tissue. Specifically, the raw Affymetrix CEL files were normalized and analyzed to obtain expression values using both dChip (<http://biosun1.harvard.edu/complab/dchip/>) and GC-RMA (50,51). Both sets of obtained expression values analyzed (as described below) and the results from the two methods were compared. We used significant analysis of

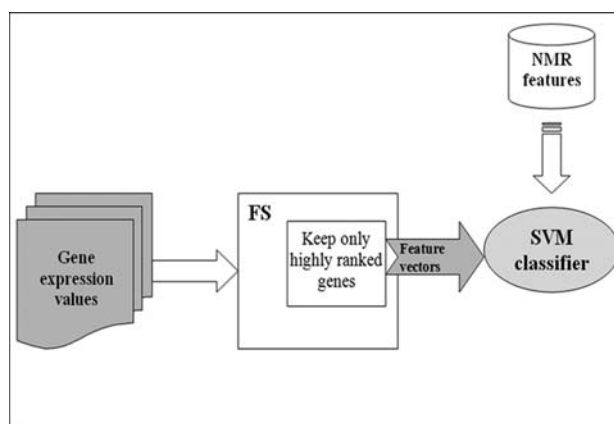


Figure 1. Classification system architecture.

microarrays (SAMs) (<http://www-stat.stanford.edu/~tibs/SAM/>) to obtain a list of differentially expressed genes with a false discovery rate (q-value) <0.05 and to properly take into account the substantial multiple comparison problem (52,53). BRB-Array Tools (<http://linus.nci.nih.gov/BRB-ArrayTools.html>) were used to classify tumor types based on expression patterns, using several major built-in methods including SVM and Bayesian classifier. BRB-Array software was used to divide samples into training and test datasets and to perform gene selection and model building in each cross-validation run of the training dataset. This cross-validation ensured an unbiased final prediction accuracy for the test dataset. The top genes selected by the classifiers were clustered and visualized in dChip. The enriched Gene Ontology and pathway groups in these top genes were identified by dChip and Ingenuity Pathway Analysis software and correlated with pathways implicated in CNS tumorigenesis. Analysis results were produced in both tabular and graphical formats.

**Classification strategy.** The architecture of our classification system is shown in Fig. 1. We first fine-tuned the feature selection process by which the high dimensionality of the SMAs output was reduced by selecting only the most relevant genes for the classification task. Then, a classifier was constructed to these reduced feature vectors in order to optimally partition the space according to class. We chose to use the SVM classifier (54). Finally, the constructed reduced feature space from the gene expression values was combined with the NMR features in order to examine their impact on the classifier.

**Feature selection (FS).** Feature selection methods typically rank genes according to their differential expressions among phenotypes and pick the top-ranked genes. There are two general schemes for feature selection, filters and wrappers (50,55). We used the minimum redundancy - maximum relevance (MRMR) method (51), because it is a powerful framework for selecting features that capture class characteristics in a broad spectrum by reducing mutual redundancy within the feature set. Thus, it offers greater robustness and generalization properties to the reducing feature space of samples, which can significantly improve classification accuracy.



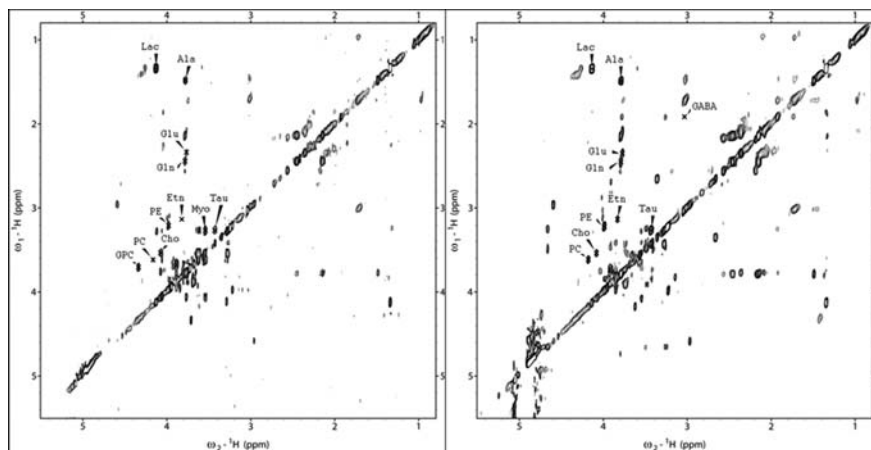


Figure 2. Typical TOTAL Through-Bond Spectroscopy (TOBSY) using *ex vivo* HRMAS MRS on anaplastic astrocytoma (left) and meningioma (right) biopsies. HRMAS <sup>1</sup>H MR spectra using TOBSY, with 45 msec mixing time, 3 kHz MAS speed, and -8°C at 600 MHz. (Ala, alanine; Cho, choline; GABA,  $\gamma$ -aminobutyric acid; Gln, glutamine; Glu, glutamate; GPC, glycerophosphocholine; Lip, lipids; Myo, myoinositol; PC, phosphocholine; PE, phospho-ethanolamine; PUFA, polyunsaturated fatty acids; Tau, taurine). Note that Cho, PC, GPC, PE, Etn, are clearly separable here due to the use of the 2D TOBSY method. Also note that the anaplastic astrocytoma (high-grade) exhibits different MR spectrum as compared to meningioma (low-grade).

*SVM classifier.* SVM (54) is a very powerful classification method that draws hyperplanes in the feature vector space by maximizing the margin between data samples of different classes. SVM is built upon the use of kernels to construct nonlinear decision boundaries. Here, we used linear kernels and the LIBSVM environment for multi-class SVMs (52). It should be noted that during all experiments with SVM, we adopted the standard leave-one-out training/testing scheme. That is, one element of the data was used as a training set, and the left-out element was used for testing the predictive performance of the resulting classifier. The SVM soft-margin constant C was set to 10, chosen based on the results of a few runs on one training set. The results indicated that the value of this parameter was not crucial for our experimental dataset.

*Statistical analysis.* Multiple stepwise logistic regression analysis was done to evaluate whether genomic and/or HRMAS MRS data can predict clinical outcome. Maximum likelihood estimation of the logistic model provided coefficients, SEs, adjusted odds ratios, 95% confidence intervals, the likelihood ratio  $\chi^2$  test for parameters as well as sensitivity, specificity and accuracy of the prediction of the clinical outcome. Statistical analysis was conducted with the SPSS software package (version 16.0, SPSS Inc., Chicago, IL), and two-tailed P-values of <0.05 were considered statistically significant.

## Results

The impact of each of the following 16 NMR features on the classifier were examined, alanine (Ala), aspartate (Asp), choline (Cho), ethanolamine (Etn),  $\gamma$ -aminobutyric acid (GABA), glutamine (Gln), glutamate (Glu), glycerophosphocholine (GPC), lactate (Lac), lipids (Lip), myoinositol (Myo), N-acetyl aspartate (NAA), phosphocholine (PC), phospho-ethanolamine (PE), polyunsaturated fatty acids (PUFA) and taurine (Tau). Typical 2D TOBSY MR spectra are shown in Fig. 2.

*A classification strategy analysis algorithm to identify combinatorial biomarker profiles that uniquely define tumor*

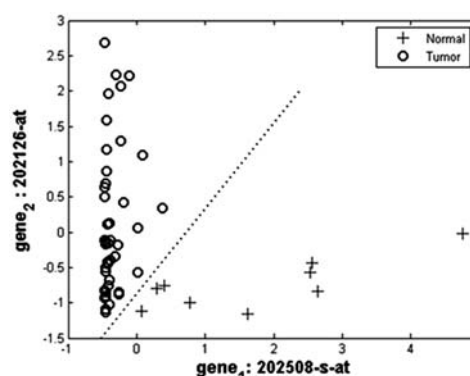


Figure 3. The rich feature space created by the two first selected genes (shown in x and y axis) that is linearly divided into normal and tumor class regions; 202126\_at is PRP4-pre-mRNA processing factor 4 (gene symbol, PRPF4B); 202508\_s\_at is synaptosomal associated protein, 25 kDa (gene symbol SNAP25). Further details on the biological function(s) of these genes and correlation with diseases are included in Table I.

*types.* Two classification problems were studied: i) distinguishing between normal and tumor classes. All 46 samples of the three tumor categories belonged to the same parent-class, tumor (two-class problem); ii) studying the different tumor categories (H, L and M) and subtypes i.e., GBM, AA, meningioma, etc. Experiments were designed for the 55-9=46 samples in an attempt to distinguish among the different types of tumors (multiple-class problem). In the two-class problem, the performance of the SVM classifier using gene values was perfect. In particular, we obtained 100% accuracy using only the first two features (genes 202126-at and 202508-s-at) that were selected by the MRMR feature selection method. As shown in Fig. 3, their discriminative ability was highly significant as they established a feature space, which could be easily divided into normal and tumor sub-regions. The ability of two other genes to discriminate between tumor (either high grade or low grade) and metastasis is summarized in Fig. 4. Gene 1552797\_s\_at is relevant to a

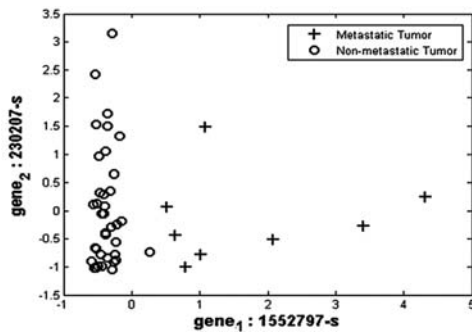


Figure 4. The rich feature space created by the two genes shown in the x and y axis with discriminatory ability between tumor and metastasis; 230207\_s\_at is dedicated to cytokinesis 5 proteing (gene symbol, DOCK5); 1552797\_s\_at is prominin 2 (gene symbol, PROM2). Further details on these genes' biological function(s) and correlation with diseases are included in Table I.

stem cell marker for malignant brain tumors, cd133 (53); iii) tumor typing and subtype classification. As expected, this classification scheme was more difficult. The classifier had near excellent behavior using the first 9-25 features selected by the MRMR method (Table I). Using the first 15 genes, the classifier reached 100% best accuracy. Specifically, after adding gene 209771\_x\_at to the feature vectors, the classification performance was 80.4%; adding gene 229851\_s\_at increased the performance to 91.3%; adding gene 225491\_at increased it to 95.6%; adding genes 211991\_s\_at, 1552797\_s\_at, 224209\_s\_at, 206349\_at, 204131\_s\_at and 241938\_at increased it to 97.8%. Finally, the addition of gene 204501\_at increased the classification performance to 100% for anaplastic astrocytoma and meningioma. We also tested the impact of combining selected NMR features (isolated or combined) with gene values. Using all 15 NMR features and selected genes (obtained from the MRMR genes selection method) increased the classification performance from 95.6% to 97.8% for the high grade typing (11 genes and 15 NMR features), from 95.6% to 100% for schwannoma subtyping (1 gene 209169\_at and 15 NMR features) and from 95.6% to 97.8% for metastasis subtyping (12 genes and 15 NMR features).

*Studies demonstrating the potential of HRMAS combined with gene expression profiles offer better accuracy than each methodology alone in predicting survival.* We performed multiple stepwise logistic regression analysis to evaluate how gene expression values, HRMAS MRS data, and their combination predict survival. We chose the 15 best genes according to their MRMR algorithm rank (Table II) and 15 metabolite values (Ala, Asp, Cho, Etn, GABA, Gln, Glu, GPC, Lac, Lip, Myo, NAA, PC, PE, PUFA, Tau), (see above) corresponding to 49 available binary clinical outcomes (33 survived vs. 16 deceased). Our preliminary results have proven that the combination of genomic and HRMAS MRS data improves the ability to predict clinical outcome. More specifically, gene data alone achieved high sensitivity, predicting 15 out of 16 deceased cases (sensitivity 94%), high specificity, predicting 32 out of 33 cases (specificity 97%), and high accuracy (96%). HRMAS MRS data had inferior sensitivity (11/16, 69%), specificity (28/32, 85%), and

accuracy (80%). Combining genomics and HRMAS MRS data logistic regression achieved a perfect classification (100% for all indices) of survived and diseased cases. Although we believe that these promising results are affected by sample size, they clearly demonstrate that the combination of gene expression and MRS data predict a clinically meaningful parameter, such as survival, better than either technique alone.

## Discussion

Our objective in this study was to use a novel approach that combines biomarkers detected with magnetic resonance spectroscopy (MRS) and molecular genomics to improve the characterization and prognostication of biospecimens in molecular medicine. We aimed to develop a useful clinical tool that uses tissue fingerprinting to aid clinicians not only in making diagnostic and treatment course decisions, but also in understanding the biology of brain malignancy subtypes in humans, an important step for novel drug development. The method is based on the development of a classification strategy analysis algorithm that combines biomarker profiles generated using high-resolution *ex vivo* MRS and whole-genome expression analyses of microscale brain tumor samples as well as features from the clinical patient database (i.e., survival). We optimized and applied *ex vivo* HRMAS <sup>1</sup>H MRS and transcriptome profiling to intact tumor biopsies that are <2 mg. We then combined these data sets to develop a classification strategy analysis algorithm to produce tissue fingerprints that accurately type these biopsies and demonstrated the potential of HRMAS and its combination with gene expression profiles to offer better accuracy than each methodology alone in predicting survival.

Herein, we demonstrated for the first time that a combined approach of using metabolite and gene expression profiles allows for more accurate discernment of tumor categories and better prediction of patient survival than either method alone. Previous data have shown that metabolites derived from brain proton MRS predict clinically meaningful parameters such as treatment response and survival of children with CNS tumors (10,56,57). Gene expression has been reported to predict outcome and survival with greater accuracy than histology (18,19). Prior studies have demonstrated that *ex vivo* MRS can be used to classify brain tumors with high sensitivity, specificity, and accuracy using only 16 metabolites, which is the highest number of metabolites detected with *in vivo* 2D approaches (22). Importantly, the agreement between *ex vivo* and *in vivo* MRS data suggests that *in vivo* 2D MRS provides a means of typing inoperable tumors in the absence of biopsies or gene expression data. It was thus important to train our algorithm (which can handle missing data) with multiple kinds of information, including both MRS and gene expression data, to increase its discriminatory capability that will also allow its application *in vivo* and specifically in cases of inoperable tumors. Although the ability to produce tissue MRS data non-invasively using 2D *in vivo* MRS would provide a considerable advantage, it is beyond the scope of the current investigation. *In vivo* MRS is clinically feasible and we have used it previously to investigate whether *ex vivo* and *in vivo* MRS data agree (17). We found that such agreement exists and thus obviates the use of *in vivo* MRS to guide *ex vivo* biopsy collection for

Table I. Genes contributing to the SVM classifier for tumor typing classification between normal and tumors and between different tumor types.

Probe Set ID	Gene symbol	Gene title	Biological process term	Disease
1552797_s_at	PROM2	prominin 2	Iron ion binding, electron carrier activity, transmembrane receptor activity, growth factor activity, role in cell death	Differential expression between chromophobe RCC and oncocytoma (66)
200853_at	H2AFZ	H2A histone family, member Z	Nucleosome assembly, chromosome organization and biogenesis	Cardiac myocyte hypertrophy (67)
201839_s_at	TACSTD1	Tumor-associated calcium signal transducer 1; aka EpCAM	Gene encodes protein for plasma membrane, integral to membrane, extracellular space, role in cell metabolism, colony formation and cell proliferation	Ovarian (68), breast (69), esophageal (70), prostate cancer metastasis
<b>202126_at</b>	PRPF4B	PRP4 pre-mRNA processing factor 4 homolog B (yeast)	mRNA processing, protein amino acid phosphorylation, RNA splicing	Diseases involving PRP4-mediated phosphorylation of KLF13 plays a role in the regulation of CCL5 expression in T lymphocytes (71)
202286_s_at	TACSTD2	Tumor-associated calcium signal transducer 2	Regulation of progression through cell cycle, cell surface receptor linked signal transduction, cell proliferation, visual perception, role in cell proliferation expressed in most human carcinomas	Gelatio-lattice corneal dystrophy type III (72,73)
<b>202508_s_at</b>	SNAP25	Synaptosomal-associated protein, 25 kDa	Neurotransmitter uptake, synaptic transmission, neurotransmitter secretion, synaptic vesicle docking during exocytosis, regulation of insulin secretion, exocytosis, axonogenesis, long-term memory, endosome transport, growth hormone secretion, sleep, positive regulation of insulin secretion, positive regulation of hormone secretion, calcium ion-dependent exocytosis of neurotransmitter, regulation of synaptogenesis, role in cell fusion, exocytosis and release	Schizophrenia (74), pituitary adenomas (75)
202748_at	GBP2	Guanylate binding protein 2, interferon-inducible	Immune response, role in cell contact growth inhibition	Esophageal squamous cell carcinomas (76)
204030_s_at	SCHIP1	Schwannomin interacting protein 1	Protein binding, protein homodimerization activity, identical protein binding	No data available
204501_at	NOV	Nephroblastoma overexpressed gene	Regulation of cell growth, role in cell migration, growth and invasion	Adrenocortical tumors, astrocytomas, multiple sclerosis (77), metastatic melanoma (78), renal cell carcinoma (79)
204587_at	SLC25A14	Solute carrier family 25 (mitochondrial carrier, brain), member 14	Transport, aerobic respiration, mitochondrial transport, role in cell transmembrane potential and mitochondrial uncoupling	Amplified in brain ischemia, schizophrenia (80-82)
205933_at	SETBP1	SET binding protein 1	Regulation of transcription, DNA-dependent	Treatment of X-linked chronic granulomatous disease (X-CGD) (83)

Table I. Continued.

Probe set ID	Gene symbol	Gene title	Biological process term	Disease
206018_at	FOXG1	Forkhead box G1	Transcription, regulation of transcription, DNA-dependent, multicellular organismal development, brain development, positive regulation of neuroblast proliferation, regulation of mitotic cell cycle, dorsal/ventral pattern formation, axon midline choice point recognition, central nervous system neuron development, forebrain development, inner ear morphogenesis, negative regulation of neuron differentiation, positive regulation of cell cycle, neuron morphogenesis during differentiation, role in cell differentiation, morphogenesis and growth	Bladder cancer (84), Rett syndrome (85), medulloblastoma (86)
208998_at	UCP2	Uncoupling protein 2 (mitochondrial, proton carrier)	Transport, mitochondrial transport, response to superoxide, proton transport, role in cell transmembrane potential, cell respiration and cell quantity	Obesity, diabetes (87,88)
209496_at	RARRES2	Retinoic acid receptor responder (tazarotene induced) 2	Retinoid metabolic process, defense response role in cell migration, transmigration and differentiation	Adrenocortical carcinoma (89), ovarian endometriosis (90), APC-mutant intestinal adenomas (91),
212274_at	LPIN1	Lipin 1	Lipid metabolic process, organ regeneration, ruffle organization and biogenesis, actin cytoskeleton reorganization, cellular response to insulin stimulus, regulation of fat cell differentiation, role in cell differentiation, enlargement and appearance	Insulin resistance (92), insulin resistance in polycystic ovary syndrome (93) obesity and lipodystrophy (94)
212486_s_at	FYN	FYN oncogene related to SRC, FGR, YES	Protein amino acid phosphorylation, calcium ion transport, protein kinase cascade, multicellular organismal development, learning, feeding behavior, interspecies interaction between organisms, T cell receptor signaling pathway, neuron migration, cell surface receptor linked signal transduction, regulation of cell shape, peptidyl-tyrosine phosphorylation, forebrain development, myelination, response to ethanol, protein amino acid autophosphorylation, activated T cell proliferation, detection of mechanical stimulus involved in sensory perception of pain, ionotropic glutamate receptor signaling pathway, role in cell proliferation, quantity and activation	Neuroblastom development and survival prediction (95), Alzheimer's disease pathogenicity (96), breast carcinomas outcome prediction (97), prostate cancer (98), <i>P. aeruginosa</i> infection (99), metastasis (100,101), catalepsy (102), kindling (103)
212565_at	STK38L	Serine/threonine kinase 38 like	Protein amino acid phosphorylation, protein kinase cascade, regulation of cellular component organization and biogenesis, protein targeting, role in cell outgrowth and invasion	No data available



Table I. Continued.

Probe set ID	Gene symbol	Gene title	Biological process term	Disease
212632_at	STX7	Syntaxin 7	Intracellular protein transport, post-Golgi vesicle-mediated transport, vacuole organization and biogenesis, vesicle-mediated transport, synaptic vesicle exocytosis, role in cell fusion and transport	Melanoma (104), adenomyosis (105)
212708_at	MSL-1	Male-specific lethal-1 homolog	A multisubunit human histone acetylase complex responsible for histone H4 lysine-16 acetylation of all cellular chromosomes	No data available
213693_s_at	MUC1	Mucin 1, cell surface associated	This gene encodes a membrane bound, glycosylated phosphoprotein, which serves a protective function by binding to pathogens and also functions in a cell signaling capacity, role in cell apoptosis, growth and lysis	Breast, prostate, colon, stomach, lung, ovary and pancreas adenocarcinomas (106), intrahepatic cholangiocarcinoma metastasis and outcome (107)
214512_s_at	SUB1	SUB1 homolog ( <i>S. cerevisiae</i> )	Transcription, regulation of transcription from RNA polymerase II promoter, regulation of transcription, DNA-dependent, role in cell apoptosis	Involved in replication of simian virus 40 and adeno-associated virus (AAV) (108,109)
218035_s_at	FLJ20273	RBM47 - RNA binding motif protein 47	RNA binding, nucleotide binding, nucleic acid binding	No data available
219368_at	NAP1L2	Nucleosome assembly protein 1-like 2	Nucleosome assembly, role in cell assembly and quantity	Colon cancer (110)
224209_s_at	GDA	Guanine deaminase	Response to stimulus, nucleobase, nucleoside, nucleotide and nucleic acid metabolic process, nervous system development	Viral hepatitis (111), learning and memory (112), <a href="http://nootropics.com/cypin/index.html">http://nootropics.com/cypin/index.html</a> , severe shock (113), myocardial infraction (114,115)
224856_at	FKBP5	FK506 binding protein 5	Protein folding, role in cell apoptosis	Glioma (116), breast tumor, cervical tumor, chondrosarcoma, colorectal tumor, esophageal tumor, gastrointestinal tumor, germ cell tumor, glioma, head and neck tumor, kidney tumor; leukemia, liver tumor, lung tumor, lymphoma, non-neoplasia, normal, ovarian tumor, pancreatic tumor, primitive neuroectodermal tumor of the CNS, prostate cancer, retinoblastoma, skin tumor, soft tissue/muscle tissue tumor, uterine tumor, breast cancer database <a href="http://www.itb.cnr.it/breastcancer/php/geneReport.php?id=2289">http://www.itb.cnr.it/breastcancer/php/geneReport.php?id=2289</a> , post-traumatic stress disorder, depression and anxiety (117,118)
225321_s_at	PILRB	Paired immunoglobulin-like type 2 receptor $\beta$	Transmembrane receptor protein tyrosine kinase signaling pathway transmembrane receptor protein tyrosine kinase activation (dimerization)	BCR-Able positive ALL (119), cancer (120), <i>S. aureus</i> -induced pneumonia (121), autoimmune disorders (122)



Table I. Continued.

Probe set ID	Gene symbol	Gene title	Biological process term	Disease
228335_at	CLDN11	Claudin 11 (oligodendrocyte transmembrane protein)	Cell adhesion, spermatogenesis, axon ensheathment, calcium-independent cell-cell adhesion, role is cell proliferation and migration	Autoimmune demyelinating disease (123), gastric cancer (124), uterine leiomyoma and cancer (105)
229273_at	SALL1	Sal-like 1 ( <i>Drosophila</i> )	Ureteric bud development, transcription regulation of transcription, DNA-dependent anatomical structure morphogenesis, inductive cell-cell signaling, role in cell apoptosis	Townes-Brocks syndrome (125)
230207_s_at	DOCK5	Dedicator of cytokinesis 5	Guanyl-nucleotide exchange factor activity	Osteosarcoma, oncogenesis (126)
232164_s_at	EPPK1	Epiplakin 1	Structural molecule activity, protein binding	Breast metastatic carcinoma (127)
235371_at	GLT8D4	Glycosyltransferase 8 domain containing 4	Carbohydrate biosynthetic process	No data available
241938_at	QKI	Quaking homolog, KH domain RNA binding (mouse)	Vasculogenesis, mRNA processing, regulation of translation, transport, multicellular organismal development, spermatogenesis, axon ensheathment, RNA splicing, cell differentiation, myelination, muscle cell differentiation, long-chain fatty acid biosynthetic process, mRNA transport, role in cell apoptosis	Schizophrenia (128), glioma (129)

Bold, classification between normal and tumor types.

Table II. Genes contributing to the SVM classifier for survival classification.

Probe set ID	Gene symbol	Gene title	Biological process term	Disease
213032_at	NFIB	Nuclear factor I/B	DNA replication and transcription, regulation of transcription, DNA-dependent, role in cell formation	Pleiomorphic adenomas (130), adipocytic tumor (131)
225423_x_at	LOC100129015	Hypothetical protein LOC100129015	Endocytosis, apoptosis, cell-cell signaling, regulation of apoptosis, role in cell apoptosis and cell migration	Parkinson's disease (132), sarcoma formation by H-Ras oncogene-transformed human fibroblasts (133)
1554168_a_a	SH3KBP1	SH3-domain kinase binding protein 1	Hyaluronan metabolic process, serine-type endopeptidase inhibitor activity, endopeptidase inhibitor activity, role in cell binding and phagocytosis	Breast cancer (134)
204987_at	ITIH2	Inter- $\alpha$ (globulin) inhibitor H2	Cell motility, cell adhesion, heart development, response to wounding, axon regeneration, multicellular organismal development, cell recognition, role in cell adhesion, apoptosis and recognition	Prostate cancer (135), cancer (136), Wagner syndrome (137),
204619_s_at	VCAN	Versican	Gene encodes protein integral to membrane	No data available
230869_at	FAM155A	Family with sequence similarity 155, member A	Immune response, role in cell contact growth inhibition	Colorectal carcinoma (138)
202748_at	GBP2	Guanylate binding protein 2, interferon-inducible	Response to retinoic acid, mesoderm development, role in cell growth	Silver-Russel Syndrome (139), leiomyoma and endometriosis (140)
202016_at	MEST	Mesoderm specific transcript homolog (mouse)	Transport	Breast cancer (141)
204324_s_at	GOLIM4	Golgi integral membrane protein 4	Carnitine biosynthetic process oxidation reduction	Refsun disease (142)
205363_at	BBOX1	Butyrobetaine ( $\gamma$ ), 2-oxoglutarate dioxygenase ( $\gamma$ -butyrobetaine hydroxylase) 1	Multicellular organismal development, mitochondrial fusion, role in cell fragmentation, length and fusion	
236417_at	MFN1	Mitofusin 1	Protein targeting to mitochondrion, transport protein transport protein import into mitochondrion intracellular protein transport across a membrane	Adenocarcinoma (143,144)
222474_s_at	TOMM22	Translocase of outer mitochondrial membrane 22 homolog (yeast)	Protein amino acid phosphorylation, regulation of cell cycle, signal transduction, spermatid development, Wnt receptor signaling pathway	Colorectal cancer (145), kidney delayed graft function (DGF), (146), globozoospermia (147)
226237_at	CSNK2A2	Casein kinase 2, $\alpha$ prime polypeptide		

Table II. Continued.

Probe set ID	Gene symbol	Gene title	Biological process term	Disease
213095_x_at	AIF1	allograft inflammatory factor 1	Response to stress, inflammatory response, cell cycle arrest, negative regulation of cell proliferation, actin filament bundle formation, macrophage activation, role in cell growth, proliferation and cell cycle progression	Atherosclerosis (148,149)
218306_s_at	HERC1	Hect [homologous to the E6-AP (UBE3A) carboxyl terminus] domain and RCC1 (CHC1)-like domain (RLD) 1	Protein modification process transport	Prader-Willi/Angelman's (150)

achieving the goals of this study. In the current study, all biopsies were collected from patients undergoing surgery. Given that these *in vivo* tests are considered to be 'additional' and not 'standard' tests for patients in pain prior to their operation, it would have been ethically ill-advised to persuade parents to agree to additional tests unless such studies were medically indicated. However, if such data are available in the future, they will be provided for inclusion in our analysis, by the assisting oncologist in the clinical data collection.

We believe that *in vivo* 2D MRS will be useful for typing inoperable tumors in the absence of biopsies or gene expression data for two reasons. Firstly, *ex vivo* MRS can be used to classify brain tumors with high sensitivity, specificity, and accuracy using only 16 metabolites (22), which is the highest number of metabolites detected with *in vivo* 2D approaches. Secondly, there is agreement between the *ex vivo* and *in vivo* MRS data obtained. Accordingly, it will be critical that we train our algorithm (which is able to handle missing data) with both MRS and gene expression data as well as with other available data to increase its discriminatory capability.

We found that certain genes were useful to subtype brain tumors (Table I) and certain other genes were useful for survival classification (Table II). These genes have been reported for other cancers or diseases (see references within Tables) but are novel to brain tumors. The ability of two genes only to discriminate between two types of brain tumors (either high grade or low grade) and metastasis was excellent (Fig. 4). It is interesting that gene 1552797\_s\_at is relevant to a stem cell marker for malignant brain tumors, cd133 (53). This suggests that our work using adult brain tumor biopsies has demonstrated that with appropriate quality control, we are able to produce meaningful data and introduce a novel classification scheme that complements and substantiates the current hypothesis of cancer stem cells (53) as a means of determining brain tumor classification and treatment.

Furthermore, our work validates and extends previous work on classification strategy analysis algorithms for both *in vivo* and *in vitro* spectra from MRS (34-40). One of the principal difficulties in such analyses is the large number of metabolites that may contribute to the spectra, each with relative intensities that can greatly vary, even in samples of the same type (34). Nonetheless, even early studies reported that the spectra of body fluids obtained with MRS are systematically different between tumor patients and healthy individuals. In many cases, successful differentiation using both linear and nonlinear methods can be made based on single resonance peaks or ratios of resonance ranges (35). More recent work on brain tumors has shown that classification according to histological type and grade is possible using similar approaches, particularly linear discriminant analysis (LDA) after feature extraction with independent components analysis (ICA) in a Bayesian framework (41) or correlation analysis and stepwise LDA (36) or using belief networks (42) or using Support Vector Machines (SVMs) (22).

Also, our approach in fusing genomics and MRS to improve the typing and prognosis of human brain tumors agrees with the notion that fusion of different sources of information can improve system performance and facilitate detection, recognition, identification, tracking, change detection, and decision-making in defense, robotics, and

medicine (58,59). Some studies have previously described that classifiers have attempted to combine data from different sources (60-63). We believe that an efficient fusion scheme using complementary information can improve confidence.

Finally, results garnered from this study may lead to the development of new clinical tools to better assess operable cancers via tissue fingerprinting and to facilitate the distinction of tumor types that cannot be readily distinguished histopathologically (64) or with routine neuroimaging (65). This will enable neurooncologists, neuropathologists, neurosurgeons and neurologists to make informed decisions related to tumor type, grade, and treatment options. The results may also facilitate the clinical application of lower resolution *in vivo* MRS to inoperable cancers using metabolic biomarkers to monitor anticancer therapies, in order to improve patient survival and quality of life. More importantly, they will further elucidate the biology of brain malignancy subtypes in brain tumor patients, an important step for novel drug development. Thus, these results greatly increase the overall potential for success of future studies that combine clinical MRI, MRS and MR imaging of gene expression *in vivo* to produce improved combined images, which could then be used to readily discriminate between metastasis and high-grade gliomas, a distinction not made adequately at present (60).

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