Spinal Myxopapillary Ependymomas Demonstrate a Warburg Phenotype

Stephen C. Mack1,2,3, Sameer Agnihotri1,3, Kelsey C. Bertrand1,2,3, Xin Wang1,2,3, David J. Shih1,2,3, Hendrik Witt4,5,6, Nadia Hill1,3, Kory Zayne1,3, Mark Barszczyk1,2,3, Vijay Ramaswamy1,2,3, Marc Remke1,2,3, Yuan Thompson1,2,3, Marina Ryzhova4,5,6, Luca Massimi7, Wieslawa Grajowska8, Boleslaw Lach9, Nalin Gupta10, William A. Weiss10, Abhijit Guha1,3, Cynthia Hawkins1,2,3, Sidney Croul1,2,3, James T. Rutka1,2,3, Stefan M. Pfister4,5,6, Andrey Korshunov6,11, Melike Pekmezci10, Tarik Tihan10, Joanna J. Philips10, Nada Jabado12, Gelareh Zadeh1,3, and Michael D. Taylor1,2,3

Abstract

Purpose: Myxopapillary ependymoma (MPE) is a distinct histologic variant of ependymoma arising commonly in the spinal cord. Despite an overall favorable prognosis, distant metastases, subarachnoid dissemination, and late recurrences have been reported. Currently, the only effective treatment for MPE is gross-total resection. We characterized the genomic and transcriptional landscape of spinal ependymomas in an effort to delineate the genetic basis of this disease and identify new leads for therapy.

Experimental Design: Gene expression profiling was performed on 35 spinal ependymomas, and copy number profiling was done on an overlapping cohort of 46 spinal ependymomas. Functional validation experiments were performed on tumor lysates consisting of assays measuring pyruvate kinase M activity (PKM), hexokinase activity (HK), and lactate production.

Results: At a gene expression level, we demonstrate that spinal grade II and MPE are molecularly and biologically distinct. These are supported by specific copy number alterations occurring in each histologic variant. Pathway analysis revealed that MPE are characterized by increased cellular metabolism, associated with upregulation of HIF1α. These findings were validated by Western blot analysis demonstrating increased protein expression of HIF1α, HK2, PDK1, and phosphorylation of PDHE1A. Functional assays were performed on MPE lysates, which demonstrated decreased PKM activity, increased HK activity, and elevated lactate production.

Conclusions: Our findings suggest that MPE may be driven by a Warburg metabolic phenotype. The key enzymes promoting the Warburg phenotype: HK2, PKM2, and PDK are targetable by small-molecule inhibitors/activators, and should be considered for evaluation in future clinical trials for MPE. Clin Cancer Res; 1–9.

Introduction

Ependymoma is an incurable malignancy in 45% of patients. It can arise within the entire central nervous system and can manifest in both children and adults. While intracranial ependymomas predominate in childhood, spinal ependymomas are commonly observed from adolescence through to adulthood (1). Spinal ependymomas are generally slow-growing tumors and are divided mainly into grade I tumors, with myxopapillary histology, or grade II tumors with more classic histologic features (2). Myxopapillary ependymomas (MPE) are a distinct histologic variant, arising in specific regions of the spine including the conus medullaris, cauda equina, or filum terminale (3). Despite an overall favorable prognosis, and classification as a grade I tumor, MPE have been associated with distant metastases, subarachnoid disseminations, and late recurrences particularly in the pediatric population (4–10). Currently, the only effective treatment for MPE is complete resection, while the benefits of radiation and chemotherapy remain unclear (11). Furthermore, given the location of these tumors in the spinal cord, the morbidity associated with surgery is high, placing patients at significant risk of incontinence, impotence, and paralysis. Compared with other ependymoma subtypes, the genetic basis of MPE is poorly characterized, thereby hindering the identification of pathways and “actionable”
Translational Relevance
Myxopapillary ependymoma (MPE) is a distinct tumor entity arising predominantly in the spinal cord. Despite an overall favorable prognosis, distant metastases and late recurrences have been reported, especially in the pediatric population. Currently, the only effective treatment for MPE is a complete resection. Thus, in an effort to delineate the genetic basis of MPE, and identify novel therapeutic leads, we characterized the genomic and transcriptional landscape of 52 primary spinal ependymomas. In this study, we demonstrate that MPE may be defined by a “Warburg” phenotype as evidenced by increased protein expression of HIF1α, HK2, PDK1, and PKM2, concordant with elevated activity of numerous metabolic processes. These findings were supported functionally by decreased PKM activity, increased HK activity, and elevated lactate production. Our findings suggest that MPE may be characterized by a “Warburg” phenotype providing rationale for evaluation of glycolysis inhibitors in future clinical trials.

Materials and Methods
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Drug targets (12–15). This challenge is further complicated by a difficulty in establishing in vitro and in vivo models of MPE. To this end, we characterized the genomic (n = 46) and transcriptional (n = 35) landscape of a total of 52 primary spinal ependymomas, in an effort to elucidate the molecular underpinnings of MPE, and to identify new leads for rationale targeted therapy.

Copy number data processing and analysis
Genomic DNA and RNA from fresh frozen tumors were isolated according to the same procedures described by Witt and colleagues. Genomic DNA was hybridized to Affymetrix SNP6.0 microarrays according to the manufacturer’s instructions and preprocessed according to methods described in Witt and colleagues. Median centering of copy number probes was performed before summarization and visualization using Integrated Genome Viewer (Broad Institute, Cambridge, MA). Significant focal regions of gain or loss were identified by GISTIC2 (16).

Gene expression data processing and analysis
RNA was hybridized to Affymetrix Gene 1.0ST microarrays according to the manufacturer’s instructions. Array data was preprocessed using the same methods described by Witt and colleagues. Consensus hierarchical clustering (HCL, R package: ConsensusClusterPlus) was performed using 1,000 genes exhibiting the greatest median absolute deviation, and 5,000 genes for consensus non-negative matrix factorization (R package: NMF). Silhouette analysis was used to evaluate sample membership following consensus HCL, and SigClust was used to determine statistical significance of subgroups. A comparison was made between consensus HCL and NMF using a Rand Index, and assessed statistically by permutation of sample labels and repetition of the Rand Index calculation to generate a null distribution.

Pathway analysis of gene expression data
Gene set enrichment analysis was performed using gene sets described in Witt and colleagues, 2011 and visualized using Cytoscape: EnrichmentMap (17, 18). Single sample GSEA was also performed (Broad: GenePattern) to evaluate pathways and biologic samples over-represented in individual samples (19). A Wilcoxon rank-sum test was used, with FDR correction (Benjamini–Hochberg method), to compare the pathways/processes differentially activated between myxopapillary and grade II spinal ependymoma.

Western blot analysis
Tumor samples were lysed in PLC lysis buffer containing protease and phosphatase inhibitors (Sigma-Aldrich). Protein concentration was determined using the bicinchoninic acid assay (Thermo Fisher Scientific). Thirty micrograms of protein lysate were loaded into 10 or 12% SDS-PAGE gels. Proteins were then transferred onto polyvinylidene difluoride membrane (NEN Research Products) using a semi-dry transfer apparatus (Bio-Rad Laboratories). Membranes were blocked in 5% milk TBST or 5% BSA TBST as per manufacturer’s instructions for an hour and probed for varying proteins at 4°C overnight. See Supplementary Table S2 for dilutions and suppliers. After incubation, membranes were washed in TBST (3 × 10 minute washes) and incubated with horseradish peroxidase–conjugated antibodies against the species the primary antibody was raised against (Bio-Rad Laboratories). Protein detection and quantification was performed by using Chemiluminescence Reagent Plus (PerkinElmer) using the Alpha Imager HP imaging system for nonsaturated densitometric analysis and exposure to X-ray film.

Immunohistochemistry staining
A nonoverlapping cohort of 39 spinal ependymomas was analyzed by immunohistochemistry (IHC) for PKM2 protein expression (Schebo Bio) as previously reported (20). Tumors were assigned a score from 0 to 3 based upon the following criteria: 0, ≤5% positivity; 1, >5% but <25% positivity; 2, 25% to 75% positivity; 3, ≥75% positivity. Because our initial hypothesis was that PKM2 expression is elevated in spinal MPEs, we used a one-sided Wilcoxon rank-sum test to compare the scoring results in our independent cohort of spinal tumors analyzed by IHC.

Hexokinase and pyruvate kinase assay
Tumor samples were lysed in 100 μL of the following buffer: 50 mmol/L potassium phosphate, 2 mmol/L dithiothreitol (DTT), 2 mmol/L EDTA, and 20 mmol/L sodium fluoride. Tumor homogenate was incubated on ice for 30 minutes, followed by centrifugation at 1,000 × g at 4°C for 10 minutes. Twenty micrograms of fresh lysate was used to measure hexokinase activity using the BioVision Hexokinase Colorimetric Assay Kit (Catalog # K789-100). Twenty micrograms of fresh lysate was also used to measure pyruvate kinase activity (Catalog #K709-100).
Lactate measurements
Lactate measurements of frozen tumor samples were performed according to manufacturer's protocol and normalized to microgram lysate (Eton Bioscience).

Statistics relating to Western blots and functional assays
Western blot analysis, chemiluminescent quantification of protein, lactate measurements, hexokinase, and pyruvate kinase assays were performed in triplicate with mean and SEM reported. ANOVA was performed for multiple comparisons with post-Tukey analysis for pairwise comparisons. The Student t test was used for direct pairwise comparisons. Significance was established as \( P < 0.05 \).

Microarray data are deposited on Gene Expression Omnibus (GEO) under the identification number: GSE66787.

Results
Grade II and myxopapillary spinal ependymoma are molecularly distinct
Our cohort of 52 spinal ependymomas consisted of 24 tumors, which were histologically classified as MPE, 20 tumors as grade II ependymomas, 1 tumor as grade III ependymoma, and 7 tumors for which a histologic diagnosis was unavailable. To delineate the transcriptional heterogeneity between patients with spinal ependymoma, we performed gene expression profiling on 35 primary tumors (Supplementary Tables S1 and S3). Using two distinct unsupervised consensus clustering approaches, hierarchical clustering and non-negative matrix factorization, we demonstrate that grade II ependymoma and MPE of the spine are transcriptionally distinct (Fig. 1A–D and Supplementary Fig. S1). We next characterized the somatic copy number landscape of grade II and MPE by profiling 46 primary spinal tumors using the Affymetrix SNP 6.0 DNA microarrays. In both grade II and MPE, we determined that majority of chromosomal aberrations involved whole chromosome gains and losses, suggesting aneuploidy, while chromosomal arm and focal aberrations were less common (Fig. 2A and B). Despite convergent chromosomal gains and losses, grade II and MPE spinal ependymoma were characterized by distinct somatic copy number alterations (SCNA), with grade II ependymomas harboring specific loss of chr16 and gain of chr12, and with MPE harboring specific losses of chr2 and chr12 and gains of chr4, chr9, and chr18 (Fig. 2A and B). In addition, we found that the majority of spinal ependymomas were characterized by loss of chromosome 22, with an increase toward homozygous loss in spinal grade II versus MPE (Fig. 2A and B). In a pattern similar to pediatric ependymoma, focal copy number alterations were highly infrequent (Supplementary Fig. S2A; Supplementary Tables S4 and S5). The only focal and recurrent amplification was observed specifically in MPE (2/22 cases) encompassing the entire uncharacterized transcript C15ORF54 (Supplementary Fig. S2A and S2C). In line with

![Figure 1](https://www.aacrjournals.org/content/clincancerres/21/14/3625/F1.large.jpg)

**Figure 1.** Grade II and myxopapillary spinal ependymomas are transcriptionally distinct. A, unsupervised consensus hierarchical clustering of 35 primary spinal ependymoma gene expression profiles generated by Affymetrix Gene 1.0ST microarrays. Shown is a rank 2 classification. Significance of clustering was measured using SigClust. B, unsupervised non-negative matrix factorization of 35 spinal ependymoma gene expression profiles. Shown is a rank 2 classification. Sample overlap between consensus HCL and NMF was measured using a Rand index. C, area under the cumulative distribution function curve indicating 2 principal subgroups of spinal ependymomas. D, silhouette analysis identifies “core” representative samples within the 2 subgroups of spinal ependymoma.
previous studies, nonrecurrent statistically significant gains were observed in \textit{EGFR}, and nonrecurrent chromosomal losses were observed in \textit{AKAP12}, \textit{TGIF}, and \textit{UBB} (Supplementary Fig. S2B). We conclude that spinal grade II and MPE are ependymoma entities harboring molecularly distinct transcriptomic and SCNA profiles.

**Spinal myxopapillary ependymoma are characterized by increased gene expression of metabolic networks**

To identify the biologic processes and signaling pathways distinguishing spinal grade II and MPE, we performed gene set enrichment analysis and visualized enriched pathways using Cytoscape EnrichmentMap (14, 17, 18). We found that pathways involved in photoreceptor development, tight junction formation, and ciliogenesis/microtubule assembly defined spinal grade II ependymomas (Fig. 3A; Supplementary Table S6). In spinal MPE, we discovered an unexpected link to pathways involving angiogenesis, HIF1α/hypoxia signaling, and cellular metabolism, which represented 33% of all gene sets, enriched in the subgroup (Fig. 3A and B and Supplementary Fig. S3A and S3B; Supplementary Table S7). Using single-sample GSEA, we next attempted to identify patients who harbored elevated metabolic gene expression and whether there was a correlation with demographic parameters. Comparing between spinal MPE and grade II ependymomas, we demonstrated significant overexpression of numerous pathways involving HIF1α/hypoxia signaling, PI3K/AKT/MTOR signaling, MYC signaling, reactive oxygen species production, glycolysis, citric acid cycle, mitochondrial electron transport, and amino acid, vitamin, and lipid metabolism (Fig. 3C). Furthermore, these pathways were enriched in the youngest patients, who represent the age group associated with increased incidence of relapse and metastatic dissemination (Fig. 3C; ref. 7). We conclude that spinal MPEs are characterized by increased gene expression of metabolic networks occurring preferentially in the pediatric population and young adulthood.

**Spinal myxopapillary ependymoma are defined by a "Warburg" phenotype**

To validate the metabolic signature observed transcriptionally in spinal MPE, we performed Western blot analysis coupled with linear protein quantification by chemiluminescence. We first examined a central metabolic transcription factor, HIF1α, and demonstrated that spinal MPE exhibited increased HIF1α expression compared with spinal grade II ependymomas and adult normal spinal tissue (Fig. 4A and B and Supplementary Fig. S3A and S3B). These results were supported by increased protein expression of pyruvate dehydrogenase lipase amide kinase isozyme 1 (PDHK1), increased hexokinase 2 (HK2) expression, and decreased hexokinase 1 (HK1; Fig. 4A and B and Supplementary Fig. S4).

Given the lack of established and available cell lines, short-term cultures, and in vivo models of myxopapillary ependymoma, we used the matched primary samples to analyze the enzymatic activity levels associated with overexpression of "Warburg" signature metabolic proteins. Concordant with an increase in HK2 protein expression, we observed an increase in total HK activity specifically in spinal MPE (Fig. 4C). Together, these findings predict a shift towards elevated glycolysis and possible lactate accumulation, described as a "Warburg" phenotype (21).

Spinal myxopapillary ependymoma demonstrate a "Warburg" phenotype through elevated PKM2 expression and lactate production

The pyruvate dehydrogenase complex catalyzes the overall conversion of pyruvate to acetyl-CoA and carbon dioxide, and thereby links the glycolytic pathway to the tricarboxylic cycle. These enzymes are frequently phosphorylated and inhibited in cancers by PDK1 thus promoting the "Warburg" effect. In spinal MPE, we observed a specific increase in phosphorylation of pyruvate dehydrogenase E1α subunit (PDHE1α), thus validating the metabolic networks over-represented in Fig. 3 (Supplementary Fig. S5).

A characteristic feature of tumors exhibiting a "Warburg" phenotype is a metabolic switch from pyruvate kinase muscle isozyme isoform M1 to M2 expression. In MPE, we observed a specific increase in pyruvate kinase muscle isozone isoform M2 (PKM2) expression compared with total PKM protein levels, providing supportive evidence of a "Warburg" phenotype (2) (Fig. 5A–C).

Using immunohistochemical staining of PKM2 protein, in a nonoverlapping cohort of spinal ependymomas \((n = 39)\), we observed a significant enrichment of PKM2 expression in spinal MPE tumors (Fig. 5C). Finally, we observed a decrease in total PKM activity in spinal MPE, which overexpress PKM2 protein (Fig. 5D). This is consistent with PKM2 being a less active isoform, thereby allowing cells to accumulate metabolites for cellular growth and division (2). Finally, we demonstrate that spinal MPE exhibit increased lactate accumulation consistent with increased glycolysis and a "Warburg" phenotype (Fig. 6 and Supplementary Fig. S4).

**Discussion**

Spinal MPE although having a generally favorable prognosis, are refractory to radiotherapy, and depend largely upon surgical resection to reduce the odds of tumor relapse. Furthermore, spinal MPEs in the pediatric population have been shown to exhibit an increased incidence of relapse and metastatic dissemination (7). Given the difficulty in establishing \textit{in vitro} and \textit{in vivo} models of spinal MPE, evaluating biologic mechanisms and targets for therapy have been challenging.

In this report, we have leveraged transcriptomic and genomic technologies to examine a cohort of 52 spinal ependymomas in an effort to delineate the genetic basis of this disease, with an ultimate goal of identifying novel treatment modalities. We demonstrate that spinal grade II and MPE are histologically, transcriptionally, and genetically distinct tumor entities. While both tumors demonstrate similar patterns of whole-chromosome loss, suggesting aneuploidy, they are characterized by disparate genomic alterations with grade II tumors characterized by loss of chr16 and gain of chr12, and spinal MPE by loss of chr2 and chr12, and gain of chr4, chr9, and chr18. In line with previous genomic characterizations of ependymoma, focal and recurrent copy number alterations were rare, with the exception of \textit{C15orf54} amplification found exclusively in spinal MPE (12, 14, 22, 23). The only other amplification encompassing an entire gene, albeit occurring in a single tumor, involved \textit{EGFR}, which has been shown in previous reports to be amplified and potentially associated with poor clinical outcome in spinal MPE (24). Focal and statistically significant deletions were also observed in single tumors involving \textit{AKAP12}, \textit{TGIF}, and \textit{UBB}.
Figure 2.
Grade II and myxopapillary spinal ependymomas harbor distinct copy number landscapes. A, bar graphs of whole chromosomal gains and losses in grade II versus spinal myxopapillary ependymomas. Gains are shown in red and losses in blue. B, genome-wide view of copy number alterations in 46 spinal ependymomas generated by Affymetrix SNP6.0 DNA microarrays sub-divided by grade II and myxopapillary spinal ependymoma. Gains are shown in red and losses in blue.
Figure 3.
Pathway analysis identifies over-representation of metabolic gene sets in myxopapillary ependymoma. A, cytoscape enrichment map of significant genesets distinguishing grade II versus myxopapillary spinal ependymomas identified by GSEA and visualized in Cytoscape. A statistical significance cutoff of $P < 0.01$ and FDR $< 0.25$ was used for the pathway analysis. B, Donut plot demonstrating significant over-representation of pathways and biologic processes in involving hypoxia signaling and cellular metabolism in myxopapillary spinal ependymoma. C, single sample GSEA demonstrating subgroup-specific over-representation of pathways and biologic processes involving cellular metabolism and hypoxia signaling. Bar graph of the ages of patients is shown in the bottom plot.
Spinal grade II ependymomas harbor a variety of NF2 mutations, and in our study, were found to exhibit increased homozygous or clonal loss of chromosome 22 as compared with spinal MPE (25, 26). In addition to the genomic differences, we demonstrate that spinal grade II ependymoma and MPE are transcriptionally distinct. Specifically, spinal grade II ependymoma are characterized largely by pathways involved in ciliogenesis and microtubule assembly consistent with our previous findings (14). Conversely, we found that spinal MPE are defined by upregulation of metabolic networks involving HIF1α/hypoxia signaling, PI3K/AKT/MTOR signaling, MYC signaling, reactive oxygen species production, glycolysis, citric acid cycle, mitochondrial electron transport, and amino acid, vitamin, and lipid metabolism. These MPE-specific pathways were enriched in younger patients, who may be at greater risk of tumor dissemination. Our transcriptional findings were confirmed by increased protein expression of HIF1α, HK2, PDK1, and a decrease in HK1. Furthermore, an increase in HK2 expression was associated with elevated hexokinase activity, an indication of elevated glycolysis in spinal MPE. These proteins may represent potential avenues for drug inhibition in spinal MPE such as Lonidamine targeting hexokinase activity and Dichloroacetate targeting PDK1 (27).

HIF1α protein is a central mediator of the hypoxic response in normal cells and its expression is regulated predominantly by oxygen-dependent hydroxylation, a modification necessary for proteosomal degradation. In spinal MPE, we observed consistent upregulation of HIF1α transcript despite varied protein stability (Supplementary Fig. S3A and S3B). This suggests that the mechanisms regulating HIF1α protein stability are still active in at least a subset of spinal MPEs.

We also observed a specific increase in protein expression of PKM2 compared with total PKM levels in spinal MPE, a metabolic switch observed in tumors characterized by a “Warburg” phenotype (2, 21). This was supported by activity assays demonstrating a decrease in overall PKM activity, associated with potential accumulation of metabolites needed for macromolecule and nucleotide synthesis. PKM2 activators have also been identified, such as TEPP46 and DASA58, which may represent additional therapeutic leads for treatment of spinal MPE (ref. 28; Supplementary Fig. S6).

Our findings demonstrate that targeting tumor metabolism represents a novel therapeutic strategy for treatment of spinal MPEs. It

Figure 4.
Validation of pathway analysis characterizing a metabolic signature enriched in myxopapillary spinal ependymomas. A, Western blot analysis validation of upregulation of HIF1α, HK2, PDK1, and decreased expression of HK1. B, linear quantification of protein expression levels detected relating to proteins in A. C, increased HK2 activity in myxopapillary spinal ependymomas measured from primary tumor lysates as compared with grade II tumors (n = 12).
should also be noted that many of these agents such as lonidamine, dichloroacetate, DASA58, and TEPP46, despite promise in various preclinical cancer models, have only recently entered clinical trials and efficacy in patients is still under evaluation.

Together, our findings suggest that spinal MPE may be characterized by a Warburg phenotype as demonstrated by a specific increase in tumor lactate production. In addition, the key enzymes promoting the Warburg phenotype: HK2, PKM2, and PDK are targetable by specific small-molecule inhibitors/activators. This may represent a novel treatment strategy that should be evaluated in preclinical studies as potential therapy for spinal MPEs.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S.C. Mack, A. Guha, A. Korshunov, M.D. Taylor
Development of methodology: S.C. Mack, M.D. Taylor
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.C. Mack, S. Agnihotri, X. Wang, H. Witt, N. Hill, K. Zayne, M. Baczczyn, V. Ramaswamy, Y. Thompson, M. Ryhova, L. Massimi, W. Gajkowska, B. Lach, N. Gupta, W.A. Weiss, S.M. Pisters, A. Korshunov, J.J. Philips, M.D. Taylor
Writing, review, and/or revision of the manuscript: S.C. Mack, S. Agnihotri, X. Wang, H. Witt, Y. Thompson, N. Gupta, J.T. Rutka, S.M. Pisters, A. Korshunov, M.D. Taylor
Study supervision: J.T. Rutka, M.D. Taylor

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Figure 6.
Spinal MPE exhibit increased lactate production consistent with anaerobic glycolysis and a “Warburg” phenotype. Increased lactate production in myxopapillary spinal ependymomas measured from primary tumor lysates as compared with grade II tumors (n = 12).
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