Spinal Myxopapillary Ependymomas Demonstrate a Warburg Phenotype

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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; 21(16); 3750–8. ©2015 AACR.

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”
Spinal Myxopapillary Ependymomas Exhibit a Warburg Phenotype

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, concomitant 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

Tumor sample isolation and preparation
Clinical samples and data were utilized in accordance with research ethics board approval from both The Hospital of Sick Children (Toronto, ON, Canada) and DKFZ. Informed consent was obtained from all patients in this study. Adult and fetal spine protein samples were purchased from Biochain. Detailed patient and sample information can be found in the Supplementary Table S1.

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 methods described by Witt and colleagues. Median centering of copy number probes was performed before summarization using the Affymetrix Genome XT software.

Gene expression data processing and analysis
RNA was hybridized to Affymetrix Gene 1.0ST 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 the Affymetrix Genome XT software.

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.

Immunohistochemistry staining
A nonoverlapping cohort of 39 spinal ependymomas was analyzed by immunohistochemistry (IHC) for PKM2 protein expression (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 x 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 Chemiluminescence Reagent Plus (PerkinElmer) using the Alpha Imager HP imaging system for nonsaturated densitometric analysis and exposure to X-ray film.

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 and Supplementary Fig. S2). 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
Spinal myxopapillary ependymomas exhibit a Warburg phenotype

Spinal myxopapillary ependymomas (SME) are characterized by increased gene expression of metabolic networks.

In this report, we have leveraged transciptomic 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 C1SORF54 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 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 AKAP12, TGIF, and UBB.

Discussion

Spinal SME 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 SMEs in the pediatric population have been shown to exhibit an increased incidence of relapse and metastatic dissemination (7). Given the difficulty in establishing in vitro and in vivo models of spinal SME, 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 C1SORF54 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 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 AKAP12, TGIF, and 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 show 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 show in red and losses in blue.
Spinal Myxopapillary Ependymomas Exhibit a Warburg Phenotype

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, 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).

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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.
should also be noted that many of these agents such as lonidamine, dichloroacetate, DAS58, 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**

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Mack et al.

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