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Morphologic characteristics and immunohistochemical profile of diffuse intrinsic pontine gliomas

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Abstract

Tumors of the CNS are the second most common malignancy in children. In particular, diffuse intrinsic pontine gliomas (DIPGs) are aggressive tumors with poor prognosis and account for 10–25% of pediatric brain tumors. The majority of DIPGs are astrocytic, infiltrative and localized to the pons. Studies have shown median survival times of less than a year with 90% of children dying within 2 years. We built multi-tissue arrays with 24 post-mortem DIPG samples and analyzed the morphology and expression of several proteins (p53, EGFR, GFAP, MIB1, BMI1, B-catenin, p16, Nanog, Nestin, OCT4, OLIG2, Sox2) with the goal of identifying potential treatment targets and improving our understanding of the biology of these tumors. The majority of DIPGs were high-grade gliomas (22) with 18 cases having features of glioblastoma (WHO grade IV), and 4 cases with high-grade features consistent with anaplastic astrocytoma (WHO grade-III). One case was low grade (WHO grade II) and one case showed intermediate features between a grade II and grade III glioma (low mitotic rate, but increased cellularity and cell atypia), being difficult to grade precisely.. The majority of the tumors were positive for GFAP (24/24), MIB1 (23/24), OLIG2 (22/24), p16 (20/24), p53 (20/24), Sox2 (19/24), EGFR (16/24) and BMI1 (9/24). Our results suggest that dysregulation of EGFR and p53 may play an important role in the development of DIPGs. The majority of DIPGs express stem cell makers such as SOX2 and OLIG2, consistent with a role for tumor stem cells in the origin and maintenance of these tumors. Targeted therapies against these proteins could be beneficial in treatment.

Keywords

DIPG; gliomas; brain tumor; brainstem; pontine

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INTRODUCTION

Pediatric tumors of the central nervous system are the second most common malignancy in children.¹ Brainstem gliomas account for 10–25% of brain neoplasms in children^{2,3}, and the most common subtype, diffuse intrinsic pontine glioma (DIPG), are aggressive tumors with poor prognosis. These tumors are of astrocytic origin with an infiltrative pattern and involving the pons. The diagnosis of DIPG is based on a characteristic clinical and radiographic presentation on magnetic resonance imaging, although distinction from primitive neuroectodermal tumors (PNETs) on imaging studies alone may be difficult. There has been debate regarding the value of performing diagnostic biopsies in cases with imaging findings suggestive of DIPGs.^{4–6} Currently, biopsies are not performed in most cases, given the possibility of adverse outcomes associated with the procedure and the limited influence of the information on patient management.

Surgical removal of DIPGs is almost always not possible due to their location and relation to vital anatomic structures. Therefore, treatment strategies have traditionally been limited to radiotherapy and chemotherapy with very poor effectiveness. Most studies have shown median survival time of less than a year from diagnosis and 90% of children die within 2 years.³ A number of chemotherapeutic agents and radiotherapy strategies have been tried in the past with little success.^{3,7} Since diagnostic biopsy or surgical excision is typically not done, the tumor tissue available for research studies is extremely scarce. This has limited our understanding of the biology of these tumors, making it difficult to rationally select treatment strategies for clinical trials.

Recent literature suggests an important role for tumor stem cells in the development, progression and treatment resistance of brain gliomas. A number of genomic studies have identified various genetic abnormalities but it is not clear how these mutations alter protein expression in DIPGs.^{8,9} Regulatory factors (OCT4, SOX2 and Nanog) involved in maintaining pluripotency and self-renewal in embryonic stem cells, have been shown to be expressed in adult gliomas.^{10,11} Monje *et al.* reported the identification of a pontine precursor-like cell (PPC) population in normal human ventral pons with temporal expression coinciding with the incidence of DIPGs. This PPC population, showed expression of Nestin and OLIG2, and was proposed as a potential cell of origin for DIPGs. However, there is limited information regarding the expression of these proteins in DIPGs. Also, information on the histology and cell biology of DIPGs is limited.

We evaluated histology, and protein expression in a set of DIPGs, in order to improve our understanding of the biology of these tumors and identifying potential therapeutic targets. We built multi-tissue arrays with 24 DIPG samples obtained post-mortem and analyzed the morphology of the tumors and the expression of proteins related to stem cell phenotype (Nanog, Nestin, OCT4, OLIG2, SOX2, BMI1, -catenin), differentiation (GFAP, Synaptophysin), proliferation (MIB1) and molecular factors altered in gliomas (p53, EGFR, p16, mutant IDH1).

METHODS

Tissue Samples

Post-mortem brain tissues from 24 cases of pediatric DIPG were included. Some were from autopsies performed at the National Institutes of Health, while others were outside material submitted to our institution with limited clinical information. The study included 11 males, 9 females and 4 samples in which the gender of the patient was not known. The age at the time of death ranged from 3 to 15 years, with a median age of 7. Patient demographics are

summarized in Table 2. Two multi-tissue arrays including samples from 24 cases were constructed as previously described.¹²

Immunohistochemical Studies

Fourteen proteins of interest were selected based on published literature. Antibodies for these proteins were procured and optimized for immunohistochemistry. Table 1 summarizes the product information and dilution factors for all the antibodies used in the study. Nanog, OCT4 and SOX2 were performed manually using Dako EnVision + Mouse *in vitro* detection systems (Dako, Glostrup, Denmark) while GFAP, p53, IDH1, MIB1, p16 and synaptophysin were performed using a Ventana Benchmark autostainer (Ventana Medical Systems, Tucson, AZ) and OLIG2, BMI, Nestin and B-catenin were done in a Leica BOND-MAX autostainer (Leica Microsystems, Wetzlar, Germany). All protocols were performed according to the manufacturer's specifications. Application of the primary antibody was preceded by avidin biotin block (DAKO). Epitope retrieval modality was selected by comparing the results of retrieval on a multi-tissue control block containing 30 different tissues.

Stains were interpreted and reported based on the percent of positive cells in the tissue sample. For MIB-1, the percentage of reactive nuclei was calculated by two pathologists (MMQ and LYB) as the estimated number of positive cells divided by the estimated number of total cells multiplied by 100. Mitosis were evaluated in 50 high power fields (HPF) or until all the tissue in the array was examined. Immunohistochemical stains were scored using a scale of 1–5, where 1 = <5%, 2 = 5–25%, 3 = 25–50%, 4 = 50–75%, 5 = 75–100%.

RESULTS

Morphologic features

The majority of the cases analyzed were high-grade astrocytomas (22 cases) with 18 cases having diagnostic features of glioblastoma, WHO grade IV and 4 cases with high-grade features (mitosis, increased cellularity, high proliferation rate) consistent with anaplastic astrocytoma, WHO grade III. One case was low-grade (WHO grade II) and one case showed intermediate features between a grade II and grade III astrocytoma (low mitotic rate, but increased cellularity and cell atypia), being difficult to grade precisely. All lesions were localized to the brainstem, with one lesion extending into the cerebellum and another lesion involving the thalamus.

Although all the samples demonstrated increased cellularity relative to normal brain, the degree of cellularity varied among cases with 5 cases showing lower cellularity and 19 cases being highly cellular. Four of the five cases with low cellularity showed a low proliferative index (MIB1 labeling index less than 5%). The number of mitosis was also varied ranging from 0 – 26 in the sample represented on the tissue array (Fig. 1A). For the most part, there was a directly proportional relationship between the number of mitoses seen and the percent of MIB1 labeling. Features of glioblastoma, such as pseudo-palisading necrosis (13/24, 54%) and glomeruloid vascular proliferation (16/24, 66%), were a common finding (Fig. 1B). Microcalcifications were present in 3 of the cases (Fig. 1C), two of which also showed a minor component of gemistocytes (less than 20% of tumor cells). The tumor cells were mostly fibrillary with two cases showing giant cells (Fig. 1D) while two other lesions contained pilocytic and myxoid microcystic features in some areas, respectively. Rosenthal fibers, eosinophilic granular bodies or true angiocentric arrangement of tumor cells were not present, making a diagnosis of pilocytic astrocytoma or pilomyxoid astrocytoma unlikely. The majority of the lesions showed a clearly infiltrative pattern with tumor cells aligning along white matter tracts. For selected cases, whole-mount sections with a larger area of

tissue represented in the tissue block were prepared. These sections revealed a significant degree of intra-tumor morphologic diversity with regards to cellularity and degree of pleomorphism.

Immunohistochemical Markers

Several immunohistochemical stains were performed in the tissue array (Table 1). In all cases, the tumor cells were positive for GFAP (Fig. 2A) and negative for synaptophysin. Proliferation index assessed by MIB-1 labeling (Fig. 2B) ranged from <1% – 40% with a mean of 12%. Eleven cases showed MIB1 staining of more than 10%, and with the exception of one case (#22), the cases with high MIB1 labeling showed a significant number of mitoses. A prior study by Yoshimura *et al.* also reported high MIB-1 labeling in a series of diffuse brainstem gliomas.¹³

Selected Molecular Pathways

In an attempt to better understand the biology and molecular origin of these tumors, as well as to identify potential therapeutic targets, we analyzed the expression of several glioma-associated proteins in the 24 DIPGs. The tyrosine kinase receptor EGFR is frequently amplified and/or overexpressed in malignant gliomas^{14,15}, and sixteen of the 24 (66%) DIPG cases showed variable EGFR membranous expression (Fig. 2C). EGFR expression could be divided into cases where less than 5% of cells are positive (4 cases) or more than 30% of cells are positive (10 cases). Two cases showed intermediate positivity (between 5–29%) and 8 cases were negative. Staining for p53 showed increased staining in 20/24 (83%) cases (Fig. 2D), while p16 protein was expressed in 21/23 (91%) (Fig. 3A). Mutant IDH1 immunostains were negative in all cases (Fig. 3B).

Tumor stem cells are proposed to play a role in tumorigenesis, recurrence and resistance to treatment in brain gliomas, a subject that has received significant attention in the recent literature. We evaluated the expression of genes involved in stem cell regulation including Nestin, Nanog, SOX2 and OCT3/4 in the 24 DIPGs using IHC in the TMA. Nestin was expressed in 5/24 (21%) cases (Fig. 3C), SOX2 in 19/24 (79%) cases (Fig. 3D), and OCT3/4 in 3/24 (12%) cases (Fig. 4A). Olig2 was positive in 22/24 (92%) of cases (Fig. 4B). In contrast, all cases were negative for Nanog. We found expression of BMI1 in 6/24 (25%) DIPGs (Fig. 4C). Nuclear translocation of β -catenin has been associated with Wnt activation and cancer stem cell phenotype, but while it was expressed in 7/24 (29%) of cases, these all showed only a submembranous/cytoplasmic pattern (Fig. 4D). Nuclear staining (“translocation”) was not identified.

Discussion

The majority of children with DIPG present with a characteristic set of symptoms and classic radiographic findings. Despite this, our study shows that these tumors vary histologically and have a heterogeneous pattern of expression for various proteins. The value of performing diagnostic biopsies for cases of suspected DIPGs continues to be a matter of debate. Inter- and intra-patient morphologic variability is an important finding that must be kept in mind at the time of evaluating biopsy specimens. Also, it is important to distinguish diffuse pontine gliomas from other brainstem gliomas such as pilocytic astrocytomas, which have a much better prognosis.

OLIG2 is a transcription factor involved in development of oligodendrocytes and has been proposed to play an important role in the development of malignant gliomas from neural stem cells. In a study by Otero *et al.* looking at 90 pediatric brain tumors, all astrocytic tumors showed diffuse OLIG2 expression with glioblastomas and pilocytic astrocytomas

showing the highest expression levels.¹⁶ Recent studies have shown that the majority of DIPGs contain the H3.3 K27M mutation and express OLIG2, while pediatric supratentorial glioblastomas frequently have the H3.3 G34M mutation and do not express OLIG2. The vast majority of tumors in our DIPG TMA are positive for OLIG2, consistent with the previous reports.¹⁷ Ligon *et al.* proposed that OLIG2 is required for glioma formation.¹⁸ The proposed role of OLIG2 in glioma formation and its expression in a large percentage of DIPGs make it an attractive target for the treatment of DIPGs.

Nestin is a type VI intermediate filament protein expressed during neural embryogenesis and it has been shown to be expressed in a variety of brain tumors, including a high percentage of gliomas.¹⁹ Nestin expression has been associated with poor prognosis in gliomas and ependymomas.^{19,20} An increase in Nestin positive cells in the ventral pons that occurs as part of human development correlates temporally with the age at which most DIPGs occur.¹¹ Based on this temporal correlation, it has been proposed that DIPGs originate from a Nestin positive population of neural stem cells in the pons. Twenty-one percent of DIPGs expressed Nestin in our study, arguing that a Nestin positive cell population could give rise to DIPGs in a subset of cases. In addition, Park *et al.* suggested that Nestin is important for the survival and self renewal of neural stem cells.²¹ These findings suggest that Nestin could be a potential target for the treatment of DIPGs.

Twenty-five percent of DIPGs expressed BMI1, a polycomb protein that regulates gene expression by modifying chromatin organization. It is expressed in glioblastomas wherein it is proposed to play a role in maintaining cancer initiating stem cells.²² SOX2, OCT4 and Nanog, which are stem cell regulatory genes, have been shown to be expressed in 95% of adult gliomas in a previous study.¹⁰ In this series, higher levels of SOX2 and Nanog were associated with higher tumor grade. The majority of the samples in our TMA were high-grade tumors (grade III–IV). Although most cases were positive for SOX2, all the cases were negative for Nanog. The difference in Nanog expression between adult gliomas and the pediatric brainstem gliomas in our study is an interesting finding that highlights potentially different etiologies for adult and pediatric gliomas.

In a study by the Children's Cancer Group, 41 of 115 (~35%) malignant gliomas showed overexpression of p53.¹ Over expression of p53 protein was associated with poor prognosis as measured by the rate of progression-free survival at 5 years. In the study by Grill *et al.*, 40% of DIPGs showed mutations in p53 when analyzed by a mass spectrometric method of allele detection.⁸ Also, in the study by Louis *et al.*, 4 out of 7 brainstem gliomas demonstrated loss of portions of chromosome 17p or mutations in p53.²³ Oka *et al.* showed that 80% of DIPG cases were positive for p53 by immunohistochemistry.²⁴ In agreement with these results, 83% of cases in our study showed accumulation of p53 protein to a variable degree, with expression ranging from less than 5% in 5 cases to close to 100% in case #7. These data suggest that p53 dysregulation plays an important role in the majority of cases of DIPGs. Mutations in β -catenin, associated with alteration of Wnt signaling, have been associated with a variety of cancers. In our study, analysis of β -catenin expression in DIPG showed only submembranous/cytoplasmic staining. Nuclear localization of β -catenin, associated with the mutated form of the protein, was not observed.

A genomewide mutational analysis of glioblastomas identified mutations in IDH1 in a small subset of tumors, which subsequently was found to be present in more than 70% of diffuse gliomas of all grades and secondary glioblastomas in adults.²⁵ The presence of mutations in the *IDH1* gene correlated with a better outcome. All DIPG cases examined in our series were negative for IDH1 staining with an antibody specific for the mutant form. Although EGFR staining has been reported in pediatric gliomas, *EGFR* amplification, which is a common finding in adult high-grade astrocytomas, is a rare event in this group.²⁶ In our

TMA 16/24 (66%) of cases were positive for EGFR expression. This is in agreement with the study by Li *et al.*, where 6/11 (54%) DIPGs cases showed expression of EGFR, suggesting that this could be a potential therapeutic target in a large percentage of cases.²⁷ To this date, there is no prognostic implication associated with EGFR expression in DIPGs.

In summary, our study shows that DIPGs are heterogeneous histologically and in their protein expression, and differ from adult high-grade gliomas. Our data also demonstrates that the majority of diffuse intrinsic pontine gliomas are high-grade gliomas that express SOX2, a transcription factor active during embryogenesis, and OLIG2, a regulator of progenitor cell fate, supporting the hypothesis that DIPG is a disorder of development.

Our results also suggest that dysregulation of EGFR and p53 may play an important role in the development of DIPGs. The majority of DIPGs expressed stem cell makers such as SOX2 and OLIG2, and a subset expressed Nestin, consistent with a role for tumor stem cells in the origin and maintenance of these tumors. Targeted therapies against these proteins may prove beneficial in the treatment of DIPGs in the future.

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Fig. 1. Morphologic spectrum of DIPGs. Mitosis (A), pseudopalisading necrosis and microvascular proliferation (B), microcalcifications (C), and giant cells (D) were observed.

Fig. 2. Immunohistochemical stains showing GFAP expression (A), high MIB1 labeling (B), EGFR expression (C) and increased p53 staining (D).

Fig. 3.

Immunohistochemical stains showing nuclear staining pattern with p16 (A). Staining with IDH1 (R132H) was negative (B). Nestin positive staining with a fibrillary pattern (C). SOX2 positive staining with a nuclear distribution (D).

Fig. 4. Immunohistochemical stains showing nuclear staining with OCT3/4 (A), OLIG2 (B), and BMI1 (C). Staining for β -catenin showed a submembranous/cytoplasmic pattern (D).

Table 1

Summary of product information and dilution factors for the antibodies used in the study.

Antibody	Source	Product No.	Dilution
GFAP	Dako	M0761	1:200
MIB-1 (Ki67)	Dako	M7240	1:200
p16	Santa Cruz	C1609	1:200
EGFR	Zymed	28-0005	1:50
Synaptophysin	Zymed	18-0130	1:100
IDH1	Dianova	DIA-H09	1:20
Nestin	Abcam	105389	1:50
OCT3/4	Santa Cruz	sc5279	1:10
Nanog	Cell Signaling	4893S	1:500
BMI-1	Cell Signaling	5364S	1:200
-catenin	Novocastra	NCL-P-CAD	1:100
SOX2	Abcam	Ab97959	1:1000
OLIG2	Milipore	AB9610	1:200
p53	Dako	M7001	1:1000

Table 2

Patient demographics and histologic characteristics of the 24 DIPG samples included in the study. Mitosis were evaluated in 50 HPF or until all the tissue in the array was examined.

Case#	Age	Sex	Grade	VP	Mitosis	Necrosis	Calcifications	Giant cells	Gemistocytes	Celularity
1	8	M	IV	X	7	X	-	-	-	high
2	7	M	IV	X	1	X	-	-	-	high
3	14	F	IV	X	3	X	-	-	-	low
4	11	M	IV	X	1	X	-	-	-	low
5	15	F	II	-	0	-	-	-	-	high
6	8	M	IV	-	5	X	-	-	-	high
7	6	M	IV	X	2	-	-	-	-	high
8	5	F	IV	X	9	X	-	-	-	high
9	13	F	IV	X	9	X	-	-	-	high
10	6	M	IV	X	8	X	-	-	-	high
11	9	F	IV	-	4-26	-	-	X	-	high
12	N/A	N/A	IV	X	7	-	-	-	-	high
13	3	F	III	-	0	-	-	-	-	high
14	N/A	N/A	IV	X	13	-	X	X	-	high
15	N/A	N/A	IV	X	6	X	-	-	-	high
16	N/A	N/A	II-III	-	1	-	-	-	-	high
17	3	M	IV	X	5	-	-	-	-	high
18	N/A	M	III	-	0	-	X	-	X	high
19	7	M	IV	X	2	X	X	-	X	low
20	4	F	III	-	0	-	-	-	-	low
21	6	F	IV	X	0	X	-	-	-	low
22	5	M	IV	X	3	X	-	-	-	high
23	13	M	III	-	0	-	-	-	-	high
24	8	F	IV	X	2	X	-	-	-	high

VP = vascular proliferation.

Table 3

Summary of immunohistochemical staining results for the 24 DIPG cases.

	BM11	IDH1	Nestin	OCT 3/4	Nanog	Sox 2	OLIG2	B-cat	GFAP	synapto	p53	pl6	MIB-1	EGFR
1	-	-	-	-	-	3	5	-	+	-	2	-	15	1
2	-	-	-	-	-	1	3	-	+	-	4	2	8	2
3	-	-	-	-	-	1	3	-	+	-	2	1	20	1
4	3	-	-	-	-	3	3	-	+	-	2	1	5	-
5	-	-	-	-	-	-	-	-	+	-	1	1	<1	3
6	5	-	4	-	-	4	4	C	+	-	5	4	15	3
7	-	-	-	-	-	-	-	-	+	-	1	1	<1	3
8	-	-	1	-	-	5	5	C	+	-	1	1	10	3
9	3	-	4	-	-	5	3	C	+	-	4	2	3	3
10	-	-	-	-	-	-	5	-	+	-	1	1	20	3
11	-	-	-	-	-	-	3	-	+	-	4	2	8	1
12	-	-	-	-	-	-	4	-	+	-	2	1	8	1
13	-	-	-	-	-	1	5	-	+	-	1	-	5	1
14	-	-	-	-	-	2	5	-	+	-	1	2	20	-
15	5	-	2	-	-	5	5	C	+	-	4	2	40	3
16	-	-	-	-	-	5	5	-	+	-	1	1	5	-
17	5	-	-	+	-	5	5	C	+	-	-	2	40	5
18	4	-	1	-	-	4	3	-	+	-	1	1	<5	2
19	2	-	-	-	-	4	4	C	+	-	4	4	3	-
20	3	-	3	+	-	5	5	C	+	-	1	1	-	4
21	-	-	-	+	-	3	4	-	+	-	-	1	3	-
22	-	-	-	-	-	2	5	-	+	-	-	1	5	-
23	-	-	-	-	-	5	5	-	+	-	-	-	15	-
24	3	-	-	-	-	3	5	-	+	-	3	1	7	-

1 = <5%, 2 = 5–25%, 3 = 25–50%, 4 = 50–75%, 5 = 75–100%.

Synapto = synaptophysin, B-cat = -catenin, C = cytoplasmic pattern.