BRAF Duplications and MAPK Pathway Activation Are Frequent in Gliomas of the Optic Nerve Proper

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Abstract
Optic pathway gliomas represent a specific subtype of astrocytoma with unique clinicopathologic and biologic properties, but studies of tumors in the optic nerve proper have been hampered by limited tissue availability. We analyzed optic nerve gliomas of 59 patients (median age, 9 years; range, 3 months–66 years; 33 female, 26 male) using formalin-fixed paraffin-embedded material in tissue microarrays. Seven patients had the clinical diagnosis of neurofibromatosis type 1 (NF1). Fluorescence in situ hybridization studies were performed for BRAF, PTEN, CDKN2A (p16), and NF1. Immunohistochemistry was performed for glial fibrillary acidic protein, phospho-ERK, and mutant IDH1 R132H protein. The BRAF duplication was present in 11 (73%) of 15 evaluable tumors, including 1 NF1 patient (1 of 4 tested; 25%). The single tumor lacking BRAF duplication or NF1 association had histologic features of a ganglioglioma. Conversely, heterozygous PTEN deletions were present in 2 (8%) of 25 evaluable cases, one of which was BRAF duplicated and the other was NF1 associated. CDKN2A and NF1 deletions were absent in all tumors tested. Phospho-ERK immunoreactivity was present in 55 (96%) of 57 tumors and was mostly strong and diffuse (80%). Only 1 case of 53 expressed IDH1 R132H. Thus, optic nerve gliomas demonstrated molecular alterations typical of pilocytic astrocytomas, including the universal presence of either BRAF duplication or NF1 association and common mitogen-activated protein kinase pathway activation but very rare mutant IDH1 expression.

Key Words: BRAF, Fluorescence in situ hybridization, Glioma, MAPK, Neurofibromatosis, Optic nerve, Pilocytic astrocytoma.

INTRODUCTION
Gliomas involving the optic nerve and other optic pathway structures (i.e. optic pathway gliomas) represent a specific subtype of astrocytoma with unique clinicopathologic and biologic properties. Optic nerve gliomas may occur in the setting of neurofibromatosis type 1 (NF1) or sporadically and affect approximately 15% of children with NF1 (1). At the pathological level, NF1-associated and sporadic optic nerve gliomas are predominantly World Health Organization grade I pilocytic astrocytomas (PAs) (2–4).

Whereas optic pathway gliomas are often considered as a group, some clinical data suggest that the site within the optic pathways at which they arise can affect their biology. Tumors in the hypothalamic region can behave aggressively, and these are often of the pilomyxoid variant (5). In contrast, it has long been recognized that tumors involving the optic nerve proper, particularly in the setting of NF1, are typically indolent and, in some instances, regress spontaneously (6). Because of the benign behavior and typical radiological appearance of optic nerve gliomas, in recent decades, they have rarely been biopsied, hampering investigations into their molecular basis.

Recent insights into the molecular mechanisms responsible for PAs have centered on the importance of mitogen-activated protein kinase (MAPK) pathway signaling. In NF1-associated tumors, the mechanism is biallelic NF1 gene inactivation (7). In sporadic PAs, the most frequent molecular alteration is a tandem duplication at chromosomal region 7q34 involving the BRAF kinase domain, which leads to a novel KIAA1149:BRAF fusion (8–11). Other alterations less commonly reported include BRAF (V600E) point mutation (12), K-RAS mutations (13), SRGAP3:RAFI fusions (13, 14), small BRAF insertions (BRAF insT) (14–16), and the recently described FAM131B:BRAF fusion mediated by an interstitial deletion (17). The common biologic effect of all these alterations is MAPK pathway activation, which is an almost universal feature of PAs. Conversely, IDH1/2 mutations are absent in almost all instances, in contrast to diffuse gliomas where they are common (18–20).
Of interest, molecular alterations in PA, including optic pathway gliomas, appear to be site dependent. For example, global gene expression profiles in PA vary according to CNS site of origin (21, 22). In addition, KIAA1549:BRAF fusions are most frequent in cerebellar PAs in many studies, with incidences ranging from 72% to 94% (8, 9, 11, 13, 17, 23, 24), whereas BRAF (V600E) is more typical of hemispheric PA (12). Although BRAF alterations are also frequent in optic pathway gliomas, with reported rates ranging from 43% to 69% (9, 17, 24–26), most of the tumors previously profiled were located in the hypothalamic region. The prevalence of BRAF alterations and MAPK pathway activation in gliomas of the optic nerve itself are therefore unclear. In this study, we took advantage of a unique historical archive containing a large number of optic nerve gliomas resected en bloc to perform targeted immunohistochemical and molecular analysis of tumors at a site from which tissue is rarely removed in current clinical practice.

MATERIALS AND METHODS

Patients and Tumor Samples

Tumors obtained from 59 patients were retrieved from the archives of the former Armed Forces Institute of Pathology (Washington, DC). In most of these cases, the optic nerve was resected en bloc, often with enucleation of the globe. Initial histopathologic evaluation was consistent with PA/low-grade astrocytoma in all cases (Fig. 1). Based on the presence of neoplastic ganglion cells, 2 cases were reclassified after central rereview by several neuropathologists as gangliogliomas. Median age at surgery was 9 years old (range, 3 months–66 years), and there were 33 females and 26 males. Five tumors demonstrated intraocular extension. Storage time for archived tissue ranged from 15 to 78 years (mean, 47 years). A tissue microarray was constructed from formalin-fixed paraffin-embedded material using 2 to 5 cores, 1 mm in diameter per tumor, with sampling of different neoplastic regions when possible. Clinical evidence of NF1 was present in 7 (19%) of 37 patients for which detailed clinical records were available. All studies were approved by institutional review boards at the participating institutions.

Immunohistochemistry

Immunohistochemical studies were performed using antibodies recognizing phospho-ERK (1:400; rabbit monoclonal antibody [D13.14.4E] XP, Cell Signaling Technology, Danvers, MA), glial fibrillary acidic protein ([GFAP] prediluted, rabbit monoclonal; Ventana, Tucson, AZ), and mutant IDH1\textsuperscript{R132H} protein (clone H09, 1:50; Dianova, Hamburg, Germany). Scoring for pERK and GFAP was performed using a 4-tiered semiquantitative scale (0 to ++++) by a single neuropathologist (Fausto J. Rodriguez). The median value of multiple scores was used. IDH1 mutant protein was scored as positive or negative.

Fluorescence In Situ Hybridization

BRAF

Fluorescence in situ hybridization (FISH) studies were performed using home brew probes targeting \textit{3’BRAF} and \textit{5’BRAF} and a commercial CEP7 probe (Abbott Molecular, Des Plaines, IL) and were scored as previously described (27).

P16, NF1, and PTEN

Commercially available probes that target \textit{CDKN2A/p16} (9p21) and \textit{PTEN} (10q23) were used, with respective control probes CEP9 and CEP10 (Abbott Molecular/Vysis). A custom-made probe targeting \textit{NF1} (17q11.2) with associated CEP17 was obtained from Empire Genomics (Buffalo, NY). Appropriate target hybridization was confirmed by hybridization with spread human metaphases. Interpretation of FISH signals was performed using previously established cutoffs (23). In brief, at least 50 nonoverlapping nuclei were enumerated per tumor, and only cases with clear probe hybridization were scored using a fluorescence microscope. Cutoffs for heterozygous deletion were defined as a target/control ratio of less than 0.80.

RESULTS

BRAF Duplication Is Frequent in Glioma of the Optic Nerve, Whereas \textit{CDKN2A}, \textit{NF1}, and \textit{PTEN} Deletions Are Rare to Absent

Only the subset of TMA cases with immunofluorescent signals sufficient for evaluation was scored, and the fact that most cases were more than 4 decades old likely limited...
the technical success rate. Of 59 cases, 26 (44%) lacked successful hybridizations with any probe; 4 (7%) cases showed successful hybridizations with 1 probe only; and 29 (49%) were successful with 2 or more probes. These findings confirm that hybridization failures/weak hybridizations were not uniform across samples. Duplication of the \textit{BRAF} kinase domain (3' portion of the gene) was the most frequent molecular alteration, present in 11 (73%) of 15 of the evaluable tumor samples (Fig. 2A). Conversely, heterozygous \textit{PTEN} deletions were present in 2 (8%) of 25 cases, whereas \textit{CDKN2A} and \textit{NF1} deletions were absent in all cases successfully tested (n = 29 and 23, respectively) (Fig. 2B–D). Both cases with \textit{PTEN} deletions lacked \textit{p16} deletions; one had a concurrent \textit{BRAF} duplication and another was associated with \textit{NF1}. Polysomies involving chromosomes 7, 9, 10, and 17 were present in 2 (13%), 2 (7%), 1 (4%), and 2 (9%) cases, respectively. Of interest, 17 of 18 cases with successful \textit{BRAF} FISH studies and/or known \textit{NF1} clinical status had either \textit{BRAF} duplication or \textit{NF1} syndrome, and the single case lacking either was histologically classified as a ganglioglioma. Of 4 patients with \textit{NF1} syndrome, 1 had a concurrent \textit{BRAF} duplication (Tables 1, 2).

**FIGURE 2.** Molecular cytogenetic findings in optic nerve glioma. (A) The main molecular cytogenetic finding in optic nerve gliomas was duplication of the 3' region of the \textit{BRAF} gene, leading to 3 red signals in neoplastic cells (overlapped with green yielding yellow signals in 2 cells). Arrow indicates the normal cell pattern for reference. (B) \textit{NF1} deletions were absent in all cases, including those obtained from patients with the clinical diagnosis of \textit{NF1}. (C) Heterozygous \textit{PTEN} deletions were present in 2 cases only; 1 of these died with progressive disease. (D) Polysomies involving several chromosomes, including Chr 9 recognized with a probe targeting \textit{p16}/\textit{CEP9}, were present in a minority of cases (original magnification: 1,000 x).

**MAPK Activation Is Frequent in Optic Nerve Glioma, Whereas Mutant IDH1 Protein Expression Is Very Rare**

Given the prolonged storage age of many of the samples, immunohistochemistry for GFAP was performed to evaluate adequacy of immunoreactivity. Staining for GFAP was preserved in 53 (95%) of 56 cases, suggesting intact immunoreactivity in most of the tumors (Fig. 3A). Cases lacking immunoreactivity for GFAP or the relevant marker were excluded from interpretation.

Immunolabeling for pERK was present in 55 (97%) of 57 cases. When present, pERK immunoreactivity was usually strong and diffuse (81%) (Fig. 3B), supporting MAPK pathway activation in the vast majority of gliomas of the optic nerve and similar to PAs in other anatomic compartments. The pERK was also positive in all cases with \textit{BRAF} duplication (11 of 11); in this group, 9 were +++ and 2 were ++. Of 7 NF1 cases, 6 demonstrated strong (+++) pERK immunoreactivity and 1 was negative. Conversely, only 1 case of 53 expressed IDH1\textsuperscript{R132H} mutant protein (Fig. 3C, D), but FISH studies failed in that case. Results are summarized in Table 1.

**Optic Nerve Gliomas Are Associated With a Relatively Favorable Outcome**

Outcome data were available for 15 patients (25%). Ten patients were alive and stable 1 to 12 years after surgery (median follow-up, 6.5 years). Two patients underwent subsequent surgeries for recurrence/progression 3 months and 48 years after the first surgery. Two patients died of perioperative complications, and 1 patient died with progressive disease 5 years after surgery, which was confirmed at autopsy. No firm associations between molecular findings and outcome were identified, although the single patient who died with progressive disease had the clinical diagnosis of NF1 as well as a heterozygous \textit{PTEN} deletion.

**DISCUSSION**

Our study supports the feasibility of using older archival material for phenotypic and molecular studies, which may be of particular use in rare tumors for which surgical excess material is frequently not available. Based on GFAP and pERK immunostaining, we demonstrate a high frequency (~95%) of antigen preservation in formalin-fixed paraffin-embedded tumor tissues. Hybridization failures for FISH studies were higher, but objective scoring was possible in almost half of the cases. A particularly high extent of failure for FISH studies was noted in cases stored more than 55 years, with only 1 case of 8 in this group providing interpretable results with 2 FISH probe sets.

The \textit{BRAF} duplications and strong pERK immunostaining were frequent, whereas \textit{PTEN}, \textit{CDKN2A} (\textit{p16}), and \textit{NF1} deletions were rare to absent. This confirms MAPK pathway activation through genetic alterations as an essential biologic feature of optic nerve gliomas, analogous to PAs arising in other anatomic sites such as the cerebellum. The main difference is the greater proportion of NF1 syndrome patients with optic nerve gliomas (19%), whereas cerebellar PAs are almost always sporadic. It is of note that essentially
<table>
<thead>
<tr>
<th>Case</th>
<th>Age, years</th>
<th>Sex</th>
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<th>Site</th>
<th>Pathology</th>
<th>Chiasm Involvement</th>
<th>NF Status-Clinical</th>
<th>BRAF FISH</th>
<th>PTEN FISH</th>
<th>p16 FISH</th>
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<td>1</td>
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<td>Cough, drowsiness, vomiting, and loss of appetite</td>
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<td>NF1 Polysomy</td>
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<td>3</td>
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<td>2</td>
<td>5</td>
<td>F</td>
<td>Painless proptosis for 3 months</td>
<td>L optic nerve</td>
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<td>—</td>
<td>NF1 Polysomy</td>
<td>Polysomy</td>
<td>Polysomy</td>
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<td>—</td>
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<td>2</td>
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<td>4</td>
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<td>M</td>
<td>Gradual blindness right &gt; left</td>
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<td>NF1 —</td>
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<td>6</td>
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<td>Exophthalmos</td>
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<td>6</td>
<td>2</td>
<td>F</td>
<td>Bilateral loss of vision, nystagmus, and optic atrophy</td>
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<td>—</td>
<td>NF1 —</td>
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<td>7</td>
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<td>M</td>
<td>3-year history of proptosis, amaurosis, exophthalmos, and a mass</td>
<td>R optic nerve</td>
<td>Optic glioma</td>
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<td>NF1 Dup</td>
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<td>5</td>
<td>M</td>
<td>—</td>
<td>Optic nerve</td>
<td>Optic glioma</td>
<td>—</td>
<td>Sporadic Dup</td>
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<td>9</td>
<td>7</td>
<td>M</td>
<td>—</td>
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<td>Optic glioma</td>
<td>—</td>
<td>Sporadic Dup</td>
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<td>Normal</td>
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<td>-</td>
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<td>10</td>
<td>1</td>
<td>F</td>
<td>Seizure, increased head circumference</td>
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<td>Sporadic Dup</td>
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<td>11</td>
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<td>12</td>
<td>30</td>
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<td>Blurred vision, visual loss, proptosis</td>
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<td>Optic glioma</td>
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<td>Sporadic Dup</td>
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<td>Normal</td>
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<td>3</td>
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<td>13</td>
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<td>Unilateral intermittent proptosis</td>
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<td>Optic glioma</td>
<td>—</td>
<td>Sporadic Dup</td>
<td>Normal</td>
<td>Normal</td>
<td>—</td>
<td>3</td>
<td>Neg</td>
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<td>14</td>
<td>6</td>
<td>F</td>
<td>—</td>
<td>Optic nerve</td>
<td>Optic glioma</td>
<td>—</td>
<td>—</td>
<td>Dup</td>
<td>Het del</td>
<td>Normal</td>
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<td>3</td>
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<tr>
<td>15</td>
<td>16</td>
<td>M</td>
<td>—</td>
<td>Optic nerve</td>
<td>Optic glioma</td>
<td>—</td>
<td>—</td>
<td>Dup</td>
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<td>16</td>
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<td>Optic nerve</td>
<td>Optic glioma</td>
<td>—</td>
<td>—</td>
<td>Dup</td>
<td>—</td>
<td>—</td>
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<tr>
<td>17</td>
<td>6</td>
<td>M</td>
<td>—</td>
<td>Optic nerve</td>
<td>Optic glioma</td>
<td>—</td>
<td>—</td>
<td>Dup</td>
<td>—</td>
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<td>18</td>
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<td>F</td>
<td>Progressive proptosis, recent pain</td>
<td>R optic nerve</td>
<td>Ganglioglioma</td>
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<td>Normal</td>
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<td>Neg</td>
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<tr>
<td>19</td>
<td>13</td>
<td>F</td>
<td>Gradual unilateral visual loss for 3 years, optic atrophy</td>
<td>R optic nerve</td>
<td>Optic glioma</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>3</td>
<td>Pos</td>
<td>3</td>
<td></td>
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</table>

Dup, duplication; F, female; FISH, fluorescence in situ hybridization; GFAP, glial fibrillary acidic protein; Het del, heterozygous deletion; IHC, immunohistochemistry; L, left; M, male; Neg, negative; NF1, neurofibromatosis type 1; R, right.
100% of optic nerve gliomas in this series had either BRAF duplication or NF1 association. The single case lacking BRAF duplication or NF1 association was more consistent with a ganglioglioma than a PA, although it also had strong pERK staining, supporting MAPK pathway activation.

The PTEN and CDKN2A (p16) deletions are frequent findings in diffusely infiltrating gliomas, particularly glioblastoma (28). Conversely, PTEN deletions were rare in our cohort of optic nerve gliomas, and CDKN2A deletions were absent. This is in agreement with prior studies showing these alterations to be relatively rare in conventional PAs (29, 30). Of note, these alterations occur at a higher rate in rare PAs with anaplasia (23), which almost never arise in the optic pathways. It is also of interest that the only tumor resulting in patient death with progressive disease in the subset of patients for whom outcome data are available had a heterozygous PTEN deletion.

NF1-associated PAs are characterized at the genetic level by homozygous inactivation of the NF1 gene, which is not a feature of PAs that arise sporadically. In most instances, BRAF alterations and NF1 syndrome are mutually exclusive. Only a single NF1-associated tumor in our cohort had a BRAF alteration, an occurrence that is rare but has been documented (13, 15, 17). We did not identify NF1 deletions by FISH in our cohort, which is consistent with small genetic alterations to be responsible for NF1 biallelic inactivation in these tumors not detectable by FISH. In fact, constitutional NF1 microdeletions occur only in a small subset of NF1 patients (5%–10%) (31).

Why NF1-related gliomagenesis favors the optic nerve and pathways has always been an enigmatic feature of the syndrome. Recent insights have been provided by murine models of NF1 and optic nerve glioma. For example, Warrington et al (32) studied the role of the tumor microenvironment in optic gliomagenesis, where non-neoplastic cells secrete factors that stimulate neoplastic glial cell growth at this specific site. Little is known regarding initiation and tumor progression in sporadic PA at this site. However, the high incidence of BRAF duplications in optic nerve PA as compared with those arising in the cortex or deep gray matter suggests differences in initiating factors possibly linked to cell of origin.

With respect to biologic behavior, recent studies have described the phenomenon of oncogene-induced senescence to limit growth in PAs. Through this mechanism, an initial oncogenic growth stimulus mediated by BRAF activation limits subsequent growth by promoting growth arrest. This is supported by the expression of markers of senescence such as p16 and activation of β-galactosidase in PA cultures and cell cultures after activated BRAF transfection (33, 34). Oncogene-induced senescence may also occur after NF1 loss (35). The specific mechanisms responsible for avoiding senescence in PA are unclear, although p16 protein loss was associated with worse overall survival in a prior study (34).

Few pathological features have been associated with outcome in tumors involving the optic pathways. The pilomyxoid astrocytoma is a unique PA variant that has a propensity to involve the hypothalamic region of young children and is associated with a worse clinical outcome (5). However, pilomyxoid astrocytomas centered in the optic nerve proper have not been described. In the setting of NF1, increased mitotic activity and infiltrative features do not seem to be associated with a worse outcome in optic nerve gliomas (2), providing further support for the concept that the optic nerve is a unique anatomic site associated with a distinct biology in glioma.

### TABLE 2. Summary of Molecular and Immunohistochemical Markers

<table>
<thead>
<tr>
<th>Marker</th>
<th>n (%)</th>
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<tr>
<td>BRAF* duplication</td>
<td>11 (73)</td>
</tr>
<tr>
<td>PTEN* deletion</td>
<td>2 (8)</td>
</tr>
<tr>
<td>CDKN2A (p16)* deletion</td>
<td>0</td>
</tr>
<tr>
<td>NF1* deletion</td>
<td>0</td>
</tr>
<tr>
<td>pERK+</td>
<td></td>
</tr>
<tr>
<td>+++</td>
<td>46 (81)</td>
</tr>
<tr>
<td>++</td>
<td>6 (11)</td>
</tr>
<tr>
<td>+</td>
<td>3 (5)</td>
</tr>
<tr>
<td>0</td>
<td>2 (3)</td>
</tr>
<tr>
<td>IDH1&lt;sup&gt;Y/R132H&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>52 (98)</td>
</tr>
<tr>
<td>Positive</td>
<td>1 (2)</td>
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*Evaluated by fluorescence in situ hybridization.
†Evaluated by immunohistochemistry using a 4-tiered semiquantitative scale (0 to ++++) by a single neuropathologist (Fausto J. Rodriguez).

### FIGURE 3. Immunohistochemical findings in optic nerve glioma.

- **A**: Preserved immunoreactivity was reflected by strong glial fibrillary acidic protein staining in most of the cases studied (original magnification: 40×).
- **B**: Strong pERK immunoreactivity, consistent with MAPK pathway activation, was present in most of the cases (original magnification: 200×) (inset: original magnification: 600×, with a negative internal vessel control).
- **C, D**: Almost all cases were negative for mutant IDH1 protein expression (C) original magnification: 200×, except for a single case with diffuse moderate reactivity (D) original magnification: 600×.

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Outcome data in a subset of patients in our cohort also emphasize this relatively good prognosis of gliomas in the optic nerve.

A major finding of our study is the evidence of MAPK activation in essentially 100% of tumors with available data. This has therapeutic implications given that inhibitors of BRAF and other pathway components are actively being tested in clinical trials, including sorafenib and AZD6244 (clinicaltrials.gov). In view of the increased morbidity associated with surgery and standard chemoradiation regimens at this site, pharmacological blockade may be an attractive approach for the optic pathway in specific cases.

In summary, our study supports an important role for BRAF duplication and MAPK pathway activation in gliomas of the optic nerve, proper, similar to PAs in the cerebellum. Future studies should provide insight into the prognostic and biologic features of tumors arising in this unique anatomic compartment and hopefully will lead to much needed targeted therapies for these patients.

ACKNOWLEDGMENT

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REFERENCES