Loss of the AP-2α Transcription Factor Is Associated with the Grade of Human Gliomas

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ABSTRACT

Purpose: The activator protein (AP)-2α transcription factor plays a crucial role in the progression of several human tumors, including malignant melanoma, prostate, and breast cancer. Loss of AP-2α results in deregulation of several genes with AP-2α binding motifs such as E-cadherin, p21WAF1, MMP-2, MCAM/MUC18, VEGF, and c-KIT. The purpose of our study was to determine AP-2α expression distribution among grades of gliomas and any possible effect on prognosis.

Experimental Design: A tissue microarray was assembled from all surgical glioma cases with available tissue samples at M.D. Anderson Cancer Center since 1986 to include 72 glioblastomas, 49 anaplastic astrocytomas, 9 low-grade astrocytoma, 37 oligodendrogliomas, 15 mixed oligoastrocytomas, 20 anaplastic mixed oligoastrocytomas, and 7 gliosarcomas. The microarray included normal brain tissue, and AP-2α expression was determined by immunohistochemistry.

Results: AP-2α expression was lost on 99% (P < 0.001) and 98% (P < 0.001) of glioblastomas and anaplastic astrocytomas, respectively, compared with grade 2 astrocytomas and normal brain, all of which (100%) maintained expression of AP-2α. The loss of AP-2α was a negative prognostic indicator within the overall category of gliomas by univariate analysis (rate ratio, 4.30; 95% confidence interval, 2.60-7.10; P < 0.001). However, there was no significant effect of loss of AP-2α expression on survival observed after adjustment for patient age, Karnofsky Performance Scale score, tumor grade, and extent of resection (rate ratio, 1.2; 95% confidence interval, 0.6-2.2; P = 0.6).

Conclusions: AP-2α expression correlates inversely with glioma grade, suggesting a direct role in glioma tumorigenicity, possibly through subsequent deregulation of target genes. Of all the previously characterized markers of progression, the loss of AP-2α would be the most common (96.2%) molecular marker as an astrocytic tumor evolves from grade 2 to 3.

INTRODUCTION

Tumor progression depends on factors intrinsic to the tumor cell, including but not limited to growth factors and their cognate receptors, extracellular matrix proteins, proteases, chemokines, and cellular adhesion molecules. The expression of these factors is influenced by the environment, microenvironment, epistasis, and genetic and epigenetic factors. The transcription factor activator protein (AP)-2α has been shown to regulate many of the genes that are involved in normal cellular hemostasis. Therefore, the loss of AP-2α expression may result in dedifferentiation, proliferation, and, eventually, metastasis or invasion.

AP-2α is a 52-kDa protein mapped to the short arm of chromosome 6 near the HLA locus (1), a common molecular abnormality found in human astrocytomas. The AP-2α protein binds to a consensus palindromic core recognition element via a DNA-binding domain located within the COOH-terminal half of the protein (2). AP-2α, which is regulated by cyclic AMP and retinoic acid (3), mediates programmed gene expression during both embryonic morphogenesis and adult cell differentiation (4).

AP-2α plays a pivotal role in regulating the expression of several genes, the products of which are involved in tumor growth and metastasis. For example, AP-2α regulates genes that are involved in proliferation, cell cycle regulation (HER-2 and p21WAF1), apoptosis (c-KIT, BCL-2, and FAS/APO-1), adhesion (MCAM/MUC18 and E-cadherin), and invasion/angiogenesis (MMP-2, PAI-1, VEGF, and PAR-1). Therefore, loss of AP-2α expression may contribute to functional changes of several gene products that in normal cells are involved in cellular proliferation and differentiation. We have shown previously that down-regulation of AP-2α results in altered c-KIT and MCAM/MUC18 expression, both of which contribute to the increased metastatic potential of human malignant melanoma cells in vitro and in vivo (5). Results of other studies have provided support for the role played by AP-2α in the progression of mammary and prostate cancer (6, 7).

Studies have suggested that several tumor markers (i.e., p53, EGFR, and PDGFR) may play a role in the transformation of human gliomas. However, these findings have not been consistent, even within WHO tumor grades. The prognostic significance of these tumor markers, especially the most common one, p53, is unclear (8–11). EGFR amplification has been shown to have prognostic value in patients with anaplastic astrocytoma and in older patients with glioblastoma.
multiforme (12). However, a definite prognostic marker for high-grade gliomas has thus far not been identified. Thus, to determine whether AP-2 expression could be involved in higher-grade gliomas, we intended to determine the level of AP-2 expression across all WHO tumor grades among a large number of human glioma surgical specimens.

MATERIALS AND METHODS

Clinical Samples. Duplicate samples from distinctly different areas of each tumor were arrayed on one slide from resected gliomas with available tissue samples between 1986 and 2001 (n = 246), which included 72 glioblastomas (grade 4), 49 anaplastic astrocytomas (grade 3), 9 low-grade astrocytoma (grade 2), 37 oligodendrogliomas (grade 2), 37 anaplastic oligodendrogliomas (grade 3), 15 mixed oligoastrocytomas (grade 2), 20 anaplastic mixed oligoastrocytomas (grade 3), and 7 gliosarcomas (grade 4) arrayed in paraffin blocks. Included on this slide were normal brain tissue (positive control) and glioma cell lines (negative control). Cross-referencing to each patient’s medical chart was possible through use of the Department of Neurosurgery clinical database and pathology records. Survival time was defined as the time from the M.D. Anderson Cancer Center surgery to death or to the most recent follow-up.

Microarray Immunohistochemistry. Sections obtained from the archived paraffin blocks were deparaffinized and steamed in Serotec target unmasking fluid (Serotec, Inc., Raleigh, NC) for antigen retrieval. Samples were blocked with endogenous peroxidase with 3% H2O2 followed by blocking with 4% fish gelatin and PBS.

Samples were incubated overnight with primary antibody diluted in blocking solution. AP-2 was detected using a 1:1,000 dilution of anti-AP-2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Competition studies with a blocking peptide (Promega, Madison, WI) showed that this polyclonal antibody was specific for AP-2 (13, 14). The primary antibody was washed with PBS and incubated with a biotinylated secondary antibody. Streptavidin-horseradish peroxidase was added followed by 3-amino-9-ethylcarbazole. The samples were counterstained with Gill’s no. 3 hematoxylin and subsequently washed with water and mounted with universal mount. Samples were considered AP-2 positive when two blinded, independent investigators verified positive nuclear staining of the arrayed tissue by microscopic evaluation. In almost all cases, positive AP-2 staining was uniform and virtually 100% of the tumors cells were positive. In the only exception to this rule, the mixed tumors, we determined a positivity was absolute and usually uniform, although exceptions occurred, especially in the mixed tumors such as the mixed oligoastrocytoma, in which the distribution was patchy. In the mixed oligoastrocytoma and the anaplastic mixed oligoastrocytoma cases, those cells with astrocytic features (fibrillary processes) were virtually 100% devoid of AP-2, and those cells with oligodendroglioma features (uniformly round nuclei with a classic “fried egg appearance”) were uniformly positive. In these cases, when >50% of the intranuclear staining were positive for AP-2 expression, the specimen was determined to be positive (Fig. 1).

Normal brain, including both white and gray matter and the cerebellum, had ubiquitous expression of intranuclear AP-2. Among the nine specimens from patients with astrocytoma, 100% maintained expression of AP-2. Among the 49 specimens from patients with anaplastic astrocytoma, only 2% maintained expression of AP-2 (P < 0.001 compared with astrocytoma). In the 72 glioblastoma specimens, only 1% maintained expression (P < 0.001 compared with astrocytoma).

RESULTS

Study Population. The median age of the patients was 42 years (range, 4-82 years). The majority of patients (97%) had a Karnofsky Performance Scale score of ≥70. Seven patients had a biopsy. Among the remaining 239 patients, the median extent of resection was 96%. The AP-2-positive group was significantly younger (median, 38 years; range, 4-64 years) compared with the AP-2-negative group (median, 44 years; range, 4-82 years; P = 0.001). The AP-2-positive group had a lower extent of resection (median, 89%; range, 19-100%) compared with the AP-2-negative group (median, 99%; range, 12-100%; P = 0.008).

AP-2 Expression Is Lost within High-grade Gliomas. In the vast majority of the samples, determination of intranuclear AP-2 positivity was absolute and usually uniform, although exceptions occurred, especially in the mixed tumors such as the mixed oligoastrocytoma, in which the distribution was patchy. In the mixed oligoastrocytoma and the anaplastic mixed oligoastrocytoma cases, those cells with astrocytic features (fibrillary processes) were virtually 100% devoid of AP-2, and those cells with oligodendroglioma features (uniformly round nuclei with a classic “fried egg appearance”) were uniformly positive. In these cases, when >50% of the intranuclear staining were positive for AP-2 expression, the specimen was determined to be positive (Fig. 1).

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astrocytoma and mixed oligodendroglioma-astrocytoma versus oligodendroglioma. In the case of gliosarcoma (n = 7), 29% still had positive expression of AP-2α (P = 0.005; Table 1).

**AP-2α Expression Is Lost within High-grade Astrocytomas Compared with High-grade Oligodendrogliomas.** Among WHO grade 3 and 4 tumors, there was a significant difference in AP-2α expression in anaplastic astrocytoma compared with anaplastic oligodendroglioma (P < 0.0001), suggesting that AP-2α has a greater roll in the progression of astrocytic-type tumors. Among WHO grade 2 tumors, there was no significant difference in AP-2α expression in astrocytoma versus oligodendroglioma (P = 0.32). Thus, AP-2α seems to be a marker of grade 3 and 4 astrocytic gliomas.

**AP-2α Expression Affects Downstream Markers.** There was a statistically significant association of AP-2α loss with overexpression of MMP-2 and VEGF. For example, in astrocytoma, none had lost expression of AP-2α and none overexpressed MMP-2 or VEGF (P < 0.0001). However, in glioblastoma, in which 99% had lost expression of AP-2α, 31% and 28% of the tumors overexpressed MMP-2 and VEGF, respectively (P < 0.0001). This statistically significant trend was observed across all grades for VEGF overexpression. Similar findings were seen with MMP-2 expression, except in anaplastic oligodendroglioma and anaplastic mixed oligoastrocytoma, in which the loss of AP-2α was not significantly associated with overexpression of MMP-2 (P = 0.63 and 0.34, respectively).

![Fig. 1 AP-2α expression within human gliomas as detected by immunohistochemical staining with anti-AP-2α on archival tissue arrays. Tissues were considered positive for AP-2α expression when two blinded, independent observers confirmed positive nuclear staining. A, normal white matter demonstrating positive AP-2α staining; B, normal neurons (gray matter) demonstrating positive AP-2α staining; C, normal cerebellum demonstrating positive AP-2α staining; D, typical AP-2α-positive oligodendroglioma; E, typical AP-2α-positive fibrillary astrocytoma (grade 2); F, typical anaplastic astrocytoma (grade 3) that did not stain positive for AP-2α; G, the only glioblastoma multiforme that maintained expression of AP-2α; H, typical glioblastoma multiforme (grade 4) that did not stain positive for AP-2α. Original magnification of all images, ×200.](https://cancerres.aacrjournals.org/content/75/24/6942.F1)

**Table 1 AP-2α expression within gliomas among WHO grades**

<table>
<thead>
<tr>
<th>Histology (WHO grade)</th>
<th>n</th>
<th>AP-2α expression, n (%)</th>
<th>95% CI*</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-grade astrocytoma (2)</td>
<td>9</td>
<td>9 (100)</td>
<td>66-100</td>
<td>—</td>
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<tr>
<td>Oligodendroglioma (2)</td>
<td>37</td>
<td>29 (78)</td>
<td>62-90</td>
<td>0.32</td>
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<tr>
<td>Mixed oligoastrocytoma (3)</td>
<td>15</td>
<td>12 (80)</td>
<td>52-96</td>
<td>0.27</td>
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<td>Anaplastic astrocytoma (3)</td>
<td>49</td>
<td>1 (2)</td>
<td>0-11</td>
<td>&lt;0.001</td>
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<td>Anaplastic oligodendroglioma (3)</td>
<td>37</td>
<td>13 (35)</td>
<td>20-53</td>
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<tr>
<td>Anaplastic mixed oligoastrocytoma (3)</td>
<td>20</td>
<td>12 (60)</td>
<td>36-81</td>
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<tr>
<td>Glioblastoma (4)</td>
<td>72</td>
<td>1 (1)</td>
<td>0-7</td>
<td>&lt;0.001</td>
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<td>Gliosarcoma (4)</td>
<td>7</td>
<td>2 (29)</td>
<td>4-71</td>
<td>0.005</td>
</tr>
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</table>

*NOTE: A tissue array was established that contained specimens of resected tumors (encompassing all WHO grades) from patients with gliomas. The expression of AP-2α was determined by immunohistochemistry.*

*For the proportion with AP-2α expression.
†As determined by Fisher’s exact test in comparison with low-grade astrocytoma.
**AP-2α Expression Affects Survival.** As high as 109 of 167 patients with loss of AP-2α died during follow-up. The Kaplan-Meier estimate of median survival for patients in this group was 1.64 years (95% CI, 1.14-2.14; Fig. 2). Only 18 of 79 patients with AP-2α-positive tumors died during follow-up. The loss of AP-2α was a negative prognostic indicator within the overall category of gliomas by univariate analysis (rate ratio, 4.30; 95% CI, 2.60-7.10; $P < 0.001$; Table 2), however, that effect was lost after adjustment for important confounding variables such as age, Karnofsky Performance Scale, extent of surgical resection, and tumor grade (adjusted rate ratio for loss of AP-2α, 1.18; 95% CI, 0.63-2.20, $P = 0.62$). No significant differences in the rates of survival between the two AP-2α groups were seen within each tumor grade. For example, in the only glioblastoma patient whose tumor expressed AP-2α, the survival time was 19.9 months, which was not significantly different from the 18-month median survival time of the remaining AP-2α-negative-expressing glioblastoma patients. In the case of the two patients with anaplastic astrocytoma who maintained AP-2α expression, the mean survival time was 49.3 months, which compared favorably with the 35-month mean survival time in the other patients with anaplastic astrocytoma, whose tumors were AP-2α negative. However, these groups of patients are too small to allow meaningful statistical analysis. Patients with oligodendroglioma, whose tumors maintained AP-2α expression, had a mean survival time of 47.8 months, which again compared favorably with the 34.3-month survival time in patients whose oligodendroglioma were AP-2α negative, but this was not statistically significant. No significant difference was seen in mean survival time between patients with AP-2α-positive anaplastic oligodendroglioma and those with AP-2α-negative anaplastic oligodendroglioma (51 versus 50 months, respectively). In the case of the 15 patients with mixed oligoastrocytoma, a trend toward longer survival time was seen in the 12 patients whose tumors maintained AP-2α expression compared with the three patients whose tumors were AP-2α negative (60.8 versus 47.9 months, respectively). No significant differences were seen in mean survival time between patients with AP-2α-positive and those with AP-2α-negative anaplastic oligodendroglioma (47.8 versus 46.5 months, respectively).

**DISCUSSION**

Using tissue microarrays, we have shown for the first time that high-grade human gliomas are associated with the loss of expression of the AP-2α transcriptional factor, which regulates several genes, such as MCAM/MUC18, MMP-2, and c-KIT that have been shown to be important for tumor progression and invasion. Over time, lower-grade astrocytomas can acquire genetic mutations and subsequently evolve to a higher grade (secondary glioblastoma). Differential genetic pathways leading to primary and secondary glioblastoma have been elucidated. The most well-characterized tumor markers associated with progression of glioblastoma are $p53$, $MDM2$, $EGFR$, and $PDGF$. The frequency of $p53$ protein accumulation in secondary glioblastoma is >90%, whereas in primary glioblastoma expression is <35% $MDM2$ forms a complex with $p53$, thus abolishing its transcriptional activity. Immunohistochemical staining of overexpression of $MDM2$ is observed in >50% of primary glioblastomas but in <10% of secondary glioblastomas. $EGFR$ is overexpressed in ~30% to 60% of the primary glioblastoma but rarely in secondary glioblastoma (10%). Finally, $PDGF$ overexpression is present in ~60% of secondary glioblastoma (15). The expression of AP-2α strongly correlates with tumor grade and there is a ubiquitous loss between grade 2 and 3 astrocytomas. Therefore, of the previously characterized tumor markers, the loss of AP-2α would be the most common (96.2%) molecular marker as an astrocytic tumor evolves from grade 2 to 3.

Based on the loss of heterozygosity and dosage analysis in human astrocytoma, the second most common genetic abnormality was identified previously on chromosome 6p (16). Additionally, allelic imbalance on 6p was present in 57% of patients with astrocytoma (17). The transcriptional factor AP-2α has been mapped to human chromosome 6p22 (1). The discrepancy between the data from the previously published cytogenetic analyses and our tissue microarray data may be because of different sensitivities of these assays. Point mutations resulting in a nonfunctional protein would not be detected by cytogenetic analysis. Ultimately, comparisons of in situ hybridization for AP-2α with 6p alterations in the same tumor to show correlation will ultimately be necessary to definitely establish this relationship.

We have shown previously that transfection of melanoma cells lines with a dominant-negative AP-2α, which inhibits AP-2 transactivator function, results in an increase in MMP-2 expression, activity, and subsequent invasion (18). Additionally, overexpression of AP-2α in prostate cell lines resulted in down-regulation of VEGF. AP-2α was found to repress VEGF by competing with the transcriptional activator Sp3. Loss of AP-2α in prostate cancer cells increased VEGF expression (19). Similar to these previous findings, we found that gliomas that lost AP-2α were more likely to overexpress VEGF and/or MMP-2 compared with those that still maintained AP-2α expression.
with normal brain by immunohistochemistry. The up-regulation of VEGF and/or MMP-2 does not always absolutely occur with AP-2α loss, which would be expected given the fact that AP-2α is only one of several regulatory genes for MMP-2 and VEGF (20). To further elaborate on the role of AP-2α in gliomagenesis, we transfected AP-2α into the U-87 glioma cell line, but this induced apoptosis (data not shown). This would be consistent with AP-2α having a proposed role as a tumor suppressor gene.

The loss of the AP-2α transcriptional factor may represent a molecular change that can be characteristic of astrocytic tumors, analogous to the allelic loss of 1p and 19q in oligodendroglioma (21, 22). In the mixed oligoastrocytoma tumors, the loss of AP-2α may be reflective of the tumor being more “astrocytic.” The histopathologic definition of mixed oligoastrocytoma remains controversial (23); therefore, a reliable molecular marker may be useful in providing additional diagnostic criteria. Our data support the use of AP-2α expression to separate low-grade astrocytomas from higher-grade pure astrocytomas. Furthermore, the loss of AP-2α function may play a role in the transformation of human gliomas.

Despite the potential role of AP-2α in gliomagenesis, the prognostic effect of AP-2α remains unclear. This lack of prognostic importance may be due in part to the close association of the AP-2α loss and histologic grade or the effect of additional factors that have not yet been identified. The lack of prognostic effect is not surprising because other tumor markers have failed to show prognostic significance as well. For example, the role of the amplified EGFR (wild-type) and the variant (EGFRvIII) in malignant progression and effect on progression-free survival and overall survival has been debated in the literature. The amplified wild-type EGFR was not found to be an independent prognostic indicator of survival in several studies (24–26), and one study was inconclusive (27). One study did identify EGFR as a negative prognostic indicator in younger patients (28), and several others found EGFR to be an independent unfavorable predictor of survival (12, 29, 30). The presence of the mutated EGFR, EGFRvIII, was found to be an independent and significant unfavorable prognosticator of survival (31). Further-

<table>
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<th>Characteristics</th>
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<th>Multivariate analysis</th>
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<td></td>
<td>Median, y</td>
<td>Rate ratio</td>
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<td>(95% CI)</td>
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<td>(95% CI)</td>
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<tr>
<td>AP-α status</td>
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<tr>
<td>Negative</td>
<td>1.64 (1.14-2.14)</td>
<td>4.30 (2.60-7.10)</td>
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<td>Positive*</td>
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<td>4.05 (2.53-5.58)</td>
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<td>4.88 (2.44-7.32)</td>
<td>5.00 (2.37-10.55)</td>
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<td>1.00</td>
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</table>

**NOTE:** Among anaplastic mixed oligoastrocytoma (n = 20), rate ratio (95% CI) for AP-2α negative compared with AP-2α positive is 1.43 (0.38-5.35; P = 0.60).

*Reference group (i.e., category that others are compared with; rate ratio > 1, faster rate of death; rate ratio < 1, slower rate of death; rate ratio = 1, same rate of death as reference).

**ACKNOWLEDGMENTS**

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