

AJAP1 expression modulates glioma cell motility and correlates with tumor growth and survival

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Abstract. Glioblastoma multiforme (GBM) is one of the most common primary malignant brain tumors. Unraveling the molecular and genetic complexity that determines GBM's pronounced migratory property could provide new options for therapeutic targeting that may significantly complement current surgical and chemoradiation therapy and alter the current poor outcome. In this study, we establish stable AJAP1 overexpressing glioma cells in order to examine *in vivo* tumor growth. We examine AJAP1 localization by confocal microscopy and AJAP1's functional effect on migration and invasion across surfaces coated with laminin. Finally, analysis of AJAP1 expression in murine xenografts and GBM primary tumors revealed its association with tumor growth and survival. Stable overexpression of AJAP1 promotes adherence, decreases invasion of glioma cells through an extracellular-like matrix, and slows migration in the presence of laminin. These observations are reversed by gene knockdown using multiple siRNAs. Additionally, overexpression of AJAP1 decreases colony formation in glioma cells, and leads to smaller tumor growth with increased survival in glioma xenograft mice. Loss of AJAP1 protein expression predicts worse survival in GBM patients. AJAP1 overexpression decreases cell motility in the presence of laminin and decreases tumor growth in xenografts. Its loss of expression predicts worse survival in patients. This study extends our prior observations and implicates AJAP1 as a potential prognostic marker and a viable target for therapeutic intervention in GBM.

Introduction

Glioblastoma multiforme (GBM) is one of the most common types of primary intracranial tumors that occur in adults (1), accounting for up to 30% of all brain tumors. As GBM is a malignant glioma, the prognosis for this disease remains universally dismal, in spite of modern diagnostics, sophisticated surgical techniques, and modern chemoradiotherapies. The median survival of GBM patients treated with the current standard of care, consisting of >98% tumor mass removal followed by concurrent radiation treatment and chemotherapy with the alkylating agent temozolomide, only reaches 14 (13-16) months (2). The clinical outcome of GBM patients has improved only marginally during the past few decades of intensive investigation in all therapeutic modalities, surgery, chemotherapy, radiotherapy, and immunotherapies (3). Surgical treatment alone is able to provide only approximately 8 months of survival to the patients due to the ability of GBM tumor cells to rapidly infiltrate the neuropil and to migrate, even to sites distant from the original tumor focus. Given the short median survival for GBM patients who have been treated by multimodal approaches (4), any improvement in the clinical outcome for patients with new effective therapeutic options could greatly affect survival.

The resistance of GBM to standard therapy may be due to the multitude of heterogeneous genetic alterations related to glioma cell migration. Genetic studies of the greatest impact have consistently demonstrated a few altered signaling pathways that appear fundamentally related to GBM tumorigenesis, namely, activating events in the Akt, PI3K, Ras, PTEN, or receptor tyrosine kinase pathways (such as VEGFR, EGFR, and PDGFR) and decreased activity in the RB1, p16, or TP53 signaling cascades (5,6). Some of these pathways have been implicated in the ability of cells to migrate away from the original tumor focus and diffusely invade normal cerebral parenchyma; however, no single pathway or factor appears central to this process. When untreated, more than 50% of these tumors will migrate to and invade the contralateral hemisphere (7). Inhibition of this infiltrative process could greatly enhance the effect of adjunctive therapies in the current standard of care.

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The invasive and migratory process of glioma cells is a complex, dynamic, and multifactorial process, involving multiple, discrete, but presumably coordinated steps, with alterations in intracellular signaling cascades and cell surface receptors that interact with cells and extracellular matrix (ECM) factors (8,9). In GBM, various molecules have been implicated in these processes, such as ECM components, integrins, cell-cell interacting molecules such as cadherin, connexin 43, matrix metalloproteinases, Rho, Rac, FAK, and PYK2 (8,9). However, the identification of these factors has not led to therapeutic interventions that have impacted the current clinical course of GBM.

We and others recently identified altered expression of AJAPI in GBM, suggesting a potential role as a prognostic factor in GBM tumorigenesis (10,11). Previously, using multiple genome-wide studies, we found that AJAPI is deleted in up to 16% of GBM patients and its expression lost in 86-92% of GBM patients (10). AJAPI's loss of expression correlates with promoter hypermethylation (10). AJAPI was originally described as a small single transmembrane protein interacting with E-cadherin- β -catenin complexes in adherens junctions (12). Later, AJAPI was shown to be deleted or at least expressed at low levels in oligodendroglioma (13) and neuroblastoma (14). Loss of AJAPI expression has been associated with decreased invasion in HeLa cells (15). However, its restoration of expression has been suggested to decrease invasion in glioma cells (13). These studies suggest that AJAPI may modulate migration, but may function differently in different cell types and environments. In this study, we extend our prior observations of AJAPI's effect on glioma motility by stably overexpressing it in glioma cells in the presence of laminin, but more importantly in an *in vivo* model. We also extend our prior observations of national clinical databases to our own more characterized institutional patient cohort to further establish its clinical relevance.

Materials and methods

Tumor samples and cells. Tumor samples (>95% pure tumor) were obtained in accordance with an IRB-approved protocol from the Tissue Biorepository of the Preston Robert Tisch Brain Tumor Center at Duke. Normal brain tissue from patients without brain tumors was obtained at the time of autopsy and frozen at -80°C before DNA and RNA isolation. All glioma cell lines were provided by the Duke Tissue Culture Facility. Cell lines were maintained in DMEM Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS at 37°C , 5% CO_2 , without antibiotics.

RNA extraction, cDNA synthesis, and quantitative PCR. Total RNA was isolated by using an RNeasy Mini kit (Qiagen, Valencia, CA, USA). Next, 200 ng of total RNA was reverse-transcribed by using an iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). Amplification of cDNA was monitored by following a SYBR Green protocol (Sigma, St. Louis, MO, USA) with a 7900HT Fast Real-time PCR System (Applied Biosystems, Foster City, CA, USA). PCR was initiated with a first denaturation step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. To avoid amplification of contaminated genomic DNA, forward and reverse primers

were designed at different exons (AJAPI forward: GTTACACAACGGAGCCTTC, reverse: CTTTGGGCACAGCAATTTT; GAPDH forward: GAAGGTGAAGGTCGGA GTCA, reverse: AATTTGCCATGGGTGGAAT). Quantitative values were obtained from the threshold cycle (Ct) number at which the increase in the signal associated with exponential growth of PCR products could first be detected by using SDS2.2.2 software (Applied Biosystems). The transcript level of AJAPI gene was normalized to that of GAPDH. As a reference, we used adult human brain RNA (BioChain Institute, Hayward, CA, USA).

Stable overexpression studies. The AJAPI cDNA was isolated from normal cortex by a PCR approach and ligated into the PCR-Blunt vector (Invitrogen). The sequence was verified at the Duke DNA Sequencing Facility and confirmed by NCBI Blast. The AJAPI cDNA was removed with *KpnI* and *HindIII* restriction enzymes and subcloned into the pEGFP-N1 expression plasmid. The pEGFP-AJAPI and pEGFP-N1 (control) expression plasmids were transfected with Lipofectamine 2000 (Invitrogen) into glioma cells. After 24 h, cells were diluted by 1:100 and grown in 10% DMEM containing 0.5 mg/ml of G418. One week later, cells were diluted by 1:10,000 again and cultured in 10% DMEM containing 0.5 mg of G418. After 2-3 weeks, multiple clones from single cells with green fluorescence were transferred to 6-well plates, grown, and tested for best expression by fluorescent microscopy and western blotting.

AJAPI knockdown studies. Two different siRNA oligonucleotides for AJAPI inhibition (AJAPI Stealth RNAi siRNA HSS148198 and AJAPI Stealth RNAi siRNA HSS148199, Invitrogen) and scrambled siRNA for the negative control were obtained (Invitrogen). A total of 2×10^5 cells were seeded in 6-well plates and grown to 50-60% confluency in 1.5 ml of culture medium before siRNA transfection. Cells were washed with PBS three times before transfection. Cells were transfected by adding Lipofectamine 2000 transfection reagent (Invitrogen) and siRNA dissolved in Opti-MEM I Reduced Serum Medium without FBS (0.5 nmol/l per well) according to manufacturer's recommendations. Cells were maintained in transfection medium overnight and subsequently in culture medium for 24, 48, and 72 h before analysis. The extent of RNA knockdown under these conditions was determined by cDNA synthesis as previously described and visualization on agarose gel. At 48 h, the loss of AJAPI was optimal, so this time was selected for procedures and applied in all knock-down experiments.

Cell proliferation. Cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, following the appropriate experiment, cells were trypsinized and seeded at a density of 1×10^3 per well in a 96-well plate 48 h after siRNA transfection. At various time-points, culture medium was replaced by 200 μl of MTT solution (0.5 mg/ml) and incubated for 4 h at 37°C and 5% CO_2 . Once the incubation was terminated and the presence of purple formazan crystals was verified by light microscopy, MTT solution was discarded and 200 μl of DMSO was added to dissolve the formazan crystals. Absorbance was measured

at 570 nm with an ELISA reader, and 8 to 12 wells were evaluated for each experimental condition.

Adhesion assay. Cell adhesion to ECM protein was measured. The 6-well plates were covered by 2 ml of PBS with laminin at a 1:1,000 ratio (a kind gift of Dr Irina Lebedeva from the Department of Neurobiology at Duke) for 1 h at room temperature. Cells from each experiment were then resuspended so that 1×10^5 cells were included in 2 ml of medium, which was added to each well, then incubated for 1 h at 37°C and 5% CO₂. The experiment was performed in triplicate. After 1 h, the medium with unattached cells was removed, and wells were washed with PBS three times. Adherent cells were stained with 0.5% crystal violet and dissolved in 0.5 ml of 33% acetic acid per well. The absorbance was then measured at 595 nm.

Transwell invasion assay. Transwell cell invasion assays were performed by using modified Boyden chambers (Transwell; Corning Inc., Corning, NY, USA). In brief, cells were cultured overnight in FBS-free medium, then trypsinized, washed, and suspended in serum-free medium at 10^6 cells/ml. Next, 250 μ l of cells was placed into the upper chambers with and without Matrigel and followed for various time points (2-24 h). In the bottom well, medium with 10% FBS was added to induce chemoattraction for the cells. Cells that migrated to the underside of the chamber were fixed with 3.7% paraformaldehyde in PBS. After cells were removed from the upper side of the chamber, the membranes were removed and stained with 0.5% crystal violet. Cells that invaded through the Matrigel onto the undersurface of the membrane were photographed and hand-counted by light microscopy, as well as optically densitized at 595 nm by an ELISA plate reader.

Wound healing migration assay. Cell migration was also analyzed by using a wound healing assay. Briefly, cells were seeded at a density of 2×10^5 per well in a 6-well plate. At the appropriate times, cell monolayers were scratched with the tip of a standard 200- μ l pipette tip. After being washed three times with PBS, cells were microphotographed (x400) and then incubated under standard conditions. Migration into the scratched area was documented at various time-points: 6, 12, 24, and 48 h after wounding. Scratch closure by migrating cells was compared in the experimental and the control cells relative to the total area of the scratches. Three to six scratches were evaluated for each experimental condition.

Western blot studies. Cells were lysed in RIPA buffer (Pierce, Rockford, IL), and protein was quantified with Coomassie Blue protein standards (Pierce). Lysed cells were mixed 1:1 with Laemmli buffer and boiled for 5 min, and then 25-50 μ g protein for each sample was equally added to individual lanes and electrophoresed onto agarose gels (NuPage, Invitrogen) for ~35 min at 186 V. Protein was transferred from gels to nitrocellulose membranes by semi-dry transfer cell (Bio-Rad) for ~30 min at 12-15 V. Membrane was blocked for 30 min in 5% nonfat dry milk TBST buffer, washed, and incubated with primary antibody at room temperature for 1-2 h. After washing 3 times with TBST buffer, membrane was incubated with secondary antibody HRP conjugated to IgG at room

temperature for 1 h. Proteins were detected with Supersignal West Femto Substrate (Pierce).

Glioma xenografts. Mice (n=10) were anesthetized i.p. with a 0.3-ml Ketamin and xylazine saline (1:10) mixture per approved animal protocol. Once the mice were sedated, they were placed in a stereotactic frame and secured with ear bars to the frame. Next, 1×10^6 cells in 10 μ l PBS were injected into each mouse brain, 1-mm lateral to bregma, 2-mm anterior to coronal suture, and 3-mm deep. A total of 1 μ l was injected over a 10-min period with a 1-min interval between each injection. Mice were marked after successful injections and immediately returned to their cage for direct observation until fully recovered from anesthesia, typically within 20 min. After completion of the study, brains were harvested en bloc, immediately submerged in 10% formalin for 24 h, and then paraffin-embedded.

Immunohistochemistry. For immunohistochemical analysis, 4- μ m sections were deparaffinized with xylene three times, washed with 100% ethanol to remove xylene, and rehydrated in a graded series of ethanol. Slides were placed in a boiling water bath for 20 min and then cooled at room temperature for 30 min. Slides were washed with water and treated with 0.3% hydrogen peroxide in methanol for 10 min. Excess moisture was removed, and tissues were encircled by ImmEdge pen (Vector Laboratories, Burlingame, CA, USA). Slides were washed in PBS and then blocked for 1 h with blocking buffer (PBS + 1% BSA + 20% horse serum). Block solution was aspirated away and primary antibody (4 μ g/ml) applied in a humidity chamber at 4°C overnight. After a PBS wash, secondary mouse antibody (1:100 diluted, Vector Laboratories) was incubated on slides for 1 h at room temperature, followed by PBS wash. ABC Standard Elite kit (Vector Laboratories, PK-6100) solution was applied to the slides for 30 min at room temperature and washed away with PBS. DAB reagent was applied to develop the slides, which were then washed with PBS, counterstained with standard H&E, and mounted with Cytoseal XYL (Richard-Allan Scientific, Kalamazoo, MI, USA).

Statistical analysis. Statistical significance was set *a priori* at $P < 0.05$. Analyses were performed with Microsoft Excel and SAS E-guide statistical packages (SAS, Inc., Cary, NC, USA).

Results

AJAPI localizes to the plasma membrane in glioma cells. We previously demonstrated frequent and marked reduction in expression of AJAPI in a large cohort of primary tumors and glioma cell lines when compared to normal brain (10). AJAPI protein expression was absent or markedly reduced in the majority of glioma cell lines tested. In our prior study, we demonstrated that targeted knock down of endogenous AJAPI in D409 could increase motility characteristics. In this study, we chose a glioma cell line with no AJAPI expression (U87; data not shown) and one with markedly reduced expression (U373; data not shown) to evaluate AJAPI's effect on motility in the presence of a common extracellular matrix (ECM) factor and more importantly, evaluate its effect *in vivo*. After subcloning the AJAPI cDNA into the pEGFP-N1 expression plasmid and

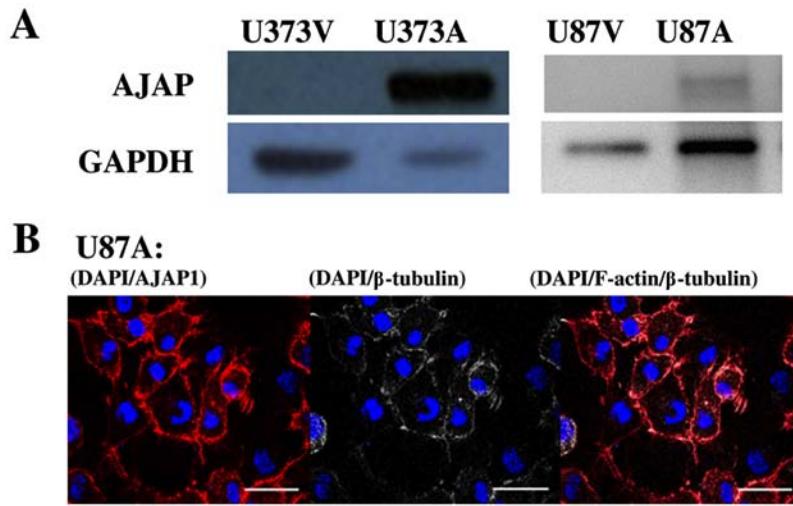


Figure 1. AJAPI is targeted to plasma membranes in glioma cells. When AJAPI is stably overexpressed in U87 and U373 glioma cells with pEGFP-AJAPI [(A) endogenous markedly reduced expression of AJAPI in U373 (data not shown) is smaller molecular weight than the AJAPI-GFP fusion protein and not reflected on this blot], it is consistently targeted to plasma membranes [(B) DAPI is blue, AJAPI is red in left panel, β -tubulin is white, and F-actin is red in the right panel]. U373V, empty plasmid; U373A, AJAPI plasmid; U87V, empty plasmid; U87A, AJAPI plasmid. F-actin and β -tubulin are also labeled to help outline cellular morphology. Bar, 10 μ m.

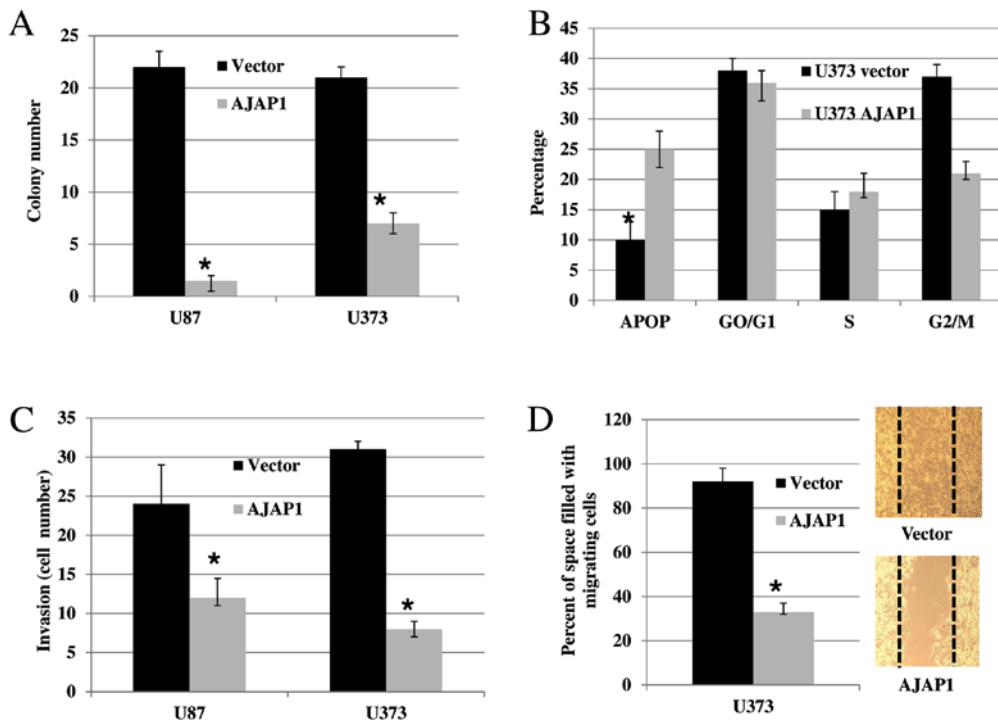


Figure 2. AJAPI overexpression decreases glioma cell colony formation, increases apoptosis, and decreases invasion and migration. Overexpression of AJAPI in U87 and U373 resulted in significantly fewer colonies after growth in soft agar for 2 weeks (A). Cell cycle FACS analysis of U373 that overexpresses AJAPI suggests that apoptotic cell death is increased, whereas cells without AJAPI have a lower apoptotic cell death rate [(B) G0/G1, S, G2/M are cell cycle stages]. Glioma cells U87 and U373 invade through a Matrigel matrix and 8- μ m membrane pore at a significantly lower rate when AJAPI is overexpressed on their surface (C). U373 cells migrate across a surface significantly less quickly with AJAPI present (D). Experiments done in triplicate. *P<0.05.

stably transfecting the plasmid into glioma cells, we confirmed robust AJAPI expression by immunoblot (Fig. 1A; endogenous markedly reduced expression of AJAPI in U373 is smaller molecular weight than the AJAPI-GFP fusion protein and not reflected on this blot). Confocal microscopy confirmed AJAPI was consistently overexpressed on the plasma membranes of glioma cells (Fig. 1B; red-tagged AJAPI in left panel; F-actin and β -tubulin are also labeled to help outline cellular

morphology in middle and right panel). We also transfected non-glioma neoplastic (MCF7) and non-neoplastic (RK3E) cells to demonstrate consistent membrane localization in other cell types (data not shown). We looked at MCF7 as a positive control, as previously seen by others (16,17). This suggests stable expression of an intact AJAPI protein that localizes to the plasma membrane in glioma cells. Protein analysis based on amino acid sequence also suggests a single transmembrane

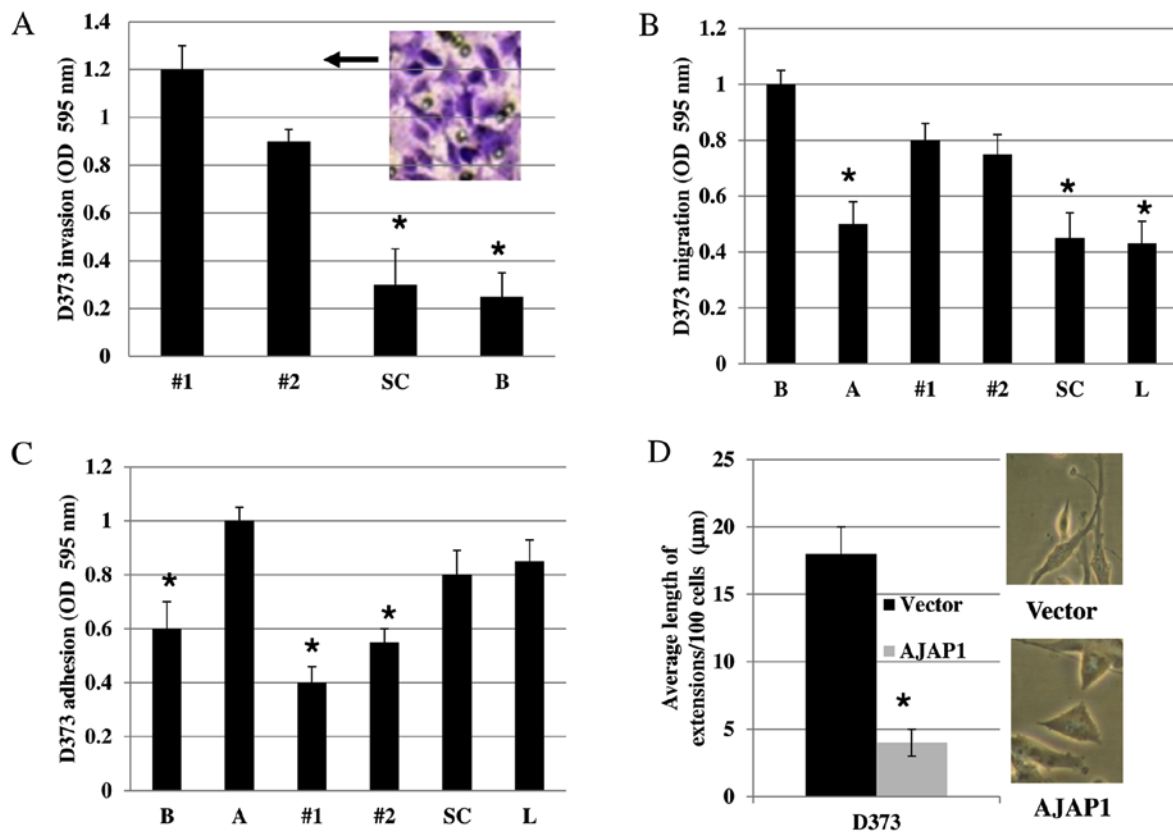


Figure 3. Loss of AJAPI expression by siRNA knockdown increases glioma cell adhesion, invasion, and migration. More D373 glioma cells invaded through Matrigel Transwell assays when AJAPI is lost by siRNA (A; siRNA#1, siRNA#2), as compared to scrambled siRNA (SC) or untreated cells (B=baseline). Insert illustrates representative membrane with cells stained blue, x40). D373 cells that have been engineered to stably overexpress AJAPI migrate less (A=AJAPI compared to B=baseline), but show increased migration after gene knockdown [#1 and #2 compared to scrambled (SC) siRNA or lipofectamine only (L) (B)]. Significantly fewer D373 cells that stably overexpressed AJAPI adhered when AJAPI was knocked down (C). D373 cells are larger, are more amoeboid-shaped, and have longer and larger extensions when AJAPI is knocked down (D). Baseline, cells alone; A=AJAPI stably transfected cells; #1, AJAPI stable transfected cells subsequently treated with siRNA#1; SC, AJAPI stably transfected cells subsequently treated with scrambled siRNA; lipo, Lipofectamine only. Experiments done in triplicate. *P<0.05.

domain with a long N-terminus extracellular domain with targeting sequence characteristics that have been studied by others in epithelial cells (18-20).

AJAPI overexpression decreases glioma cell colony formation, increases apoptosis, and decreases invasion and migration. Stable AJAPI overexpression did not significantly alter cell proliferation in our glioma cell lines, even after serum starvation (data not shown). However, overexpression of AJAPI in U87 and U373 resulted in fewer colonies after growth in soft agar for 2 weeks, an assay measuring cell anchorage-independent growth (Fig. 2A). Under normal physiologic conditions, cell death is essential for the maintenance of appropriate cell number. However, the rate of cell death may be disturbed in tumorigenesis. Cell cycle FACS analysis of U373 that overexpressed AJAPI suggests that apoptotic cell death was increased (Fig. 2B). Cells without AJAPI had a lower apoptotic cell death rate. Glioma cells invaded through a Matrigel matrix and 8- μ m membrane pore at a significantly lower rate when AJAPI was overexpressed on their surface (Fig. 2C), suggesting less ability to invade ECM-like material when AJAPI is expressed. Migration of glioma cells across a surface in a wound healing assay was significantly less with AJAPI present (Fig. 2D).

Loss of AJAPI expression by siRNA knockdown increases glioma cell invasion and migration. To ascertain the effect of the targeted loss of AJAPI expression in glioma cells, we selected a glioma cell line with AJAPI stably overexpressed (D373; Figs. 1 and 3) and knocked down expression with two AJAPI-specific siRNAs. When AJAPI was reduced by two siRNAs (Fig. 3A #1 and #2), more glioma cells invaded through Matrigel Transwell assays, in comparison to scrambled siRNA (SC) or untreated baseline cells (B). Migration was reduced in glioma cells that were engineered to stably overexpress AJAPI [Fig. 3B, baseline (B) vs. overexpressed AJAPI (A)], but was increased after AJAPI was knocked down (Fig. 3B #1 and #2). Similarly, significantly fewer glioma cells that stably overexpressed AJAPI adhered than when AJAPI was knocked down (Fig. 3C). D373 cells are larger, more amoeboid-shaped, and have longer and larger extensions when AJAPI is knocked down (Fig. 3D).

AJAPI overexpression slows tumor growth and increases survival in mice. To evaluate the role of AJAPI *in vivo*, mice (n=10/group) were stereotactically injected intracranially with U87 glioma cells stably expressing the pEGFP-N1 or pEGFP-AJAPI plasmid. As seen by immunohistochemistry (Fig. 4A, top left panel, whole mount), a typical injection

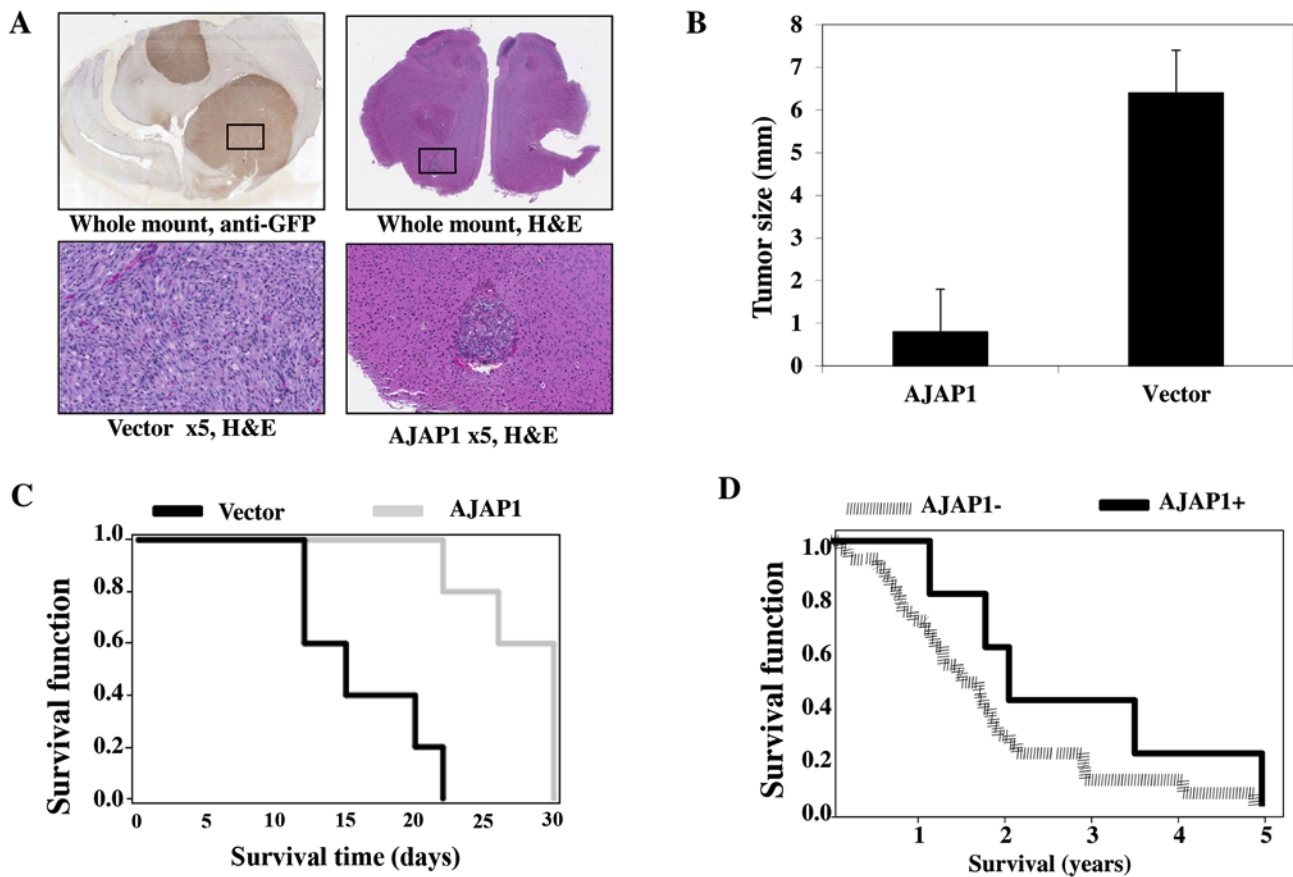


Figure 4. AJAPI overexpression slows tumor growth and increases survival in mice. Murine xenografts (n=10/group) stereotactically injected intracranially with U87 glioma cells stably expressing the pEGFP-N1 plasmid as seen by immunohistochemistry (A, top left panel, whole mount showing two large tumor nodules positive for GFP). At high magnification, U87 tumors demonstrate increased cellularity, cellular and nuclear atypia, increased mitotic images, pseudopalisading cells, and hypervascularity (A, bottom left panel). U87 tumors with AJAPI overexpression are significantly smaller (A, top right and bottom right panels). U87 gliomas that overexpress AJAPI (B, AJAPI=pEGFP-AJAPI) result in significantly smaller tumors than AJAPI-negative tumor cells (B, Vector=pEGFP-N1). Glioma xenografts that overexpressed AJAPI resulted in longer survival than AJAPI-negative cells. We evaluated a U87 xenograft with a pEGFP-N1 vector versus U87 xenograft with AJAPI overexpression via a pEGFP-AJAPI construct (C, log-rank P=0.01). *AJAPI* loss demonstrates worse survival in GBM patients (D). Patients (n=35) with markedly reduced or absent AJAPI (AJAPI-) protein expression trended toward worse survival than patients with intermediate or normal AJAPI expression (AJAPI+) (log-rank P=0.09). All patients underwent standard of care therapy, including gross total resection of visible enhancing tumor on MRI, followed by at least 6 months of concurrent radiation therapy and temozolomide. In this cohort, age <45 years predicted better survival (P<0.05). This is consistent with our prior observation of the public database Rembrandt, where all glioma patients were assessed. Those with downregulation of AJAPI expression (>2-fold loss, n=206) clearly had significantly worse survival than those with intermediate or normal expression (n=91; P=0.0056) (10).

generated 1-2 large tumor nodules positive for GFP. At high magnification, tumors demonstrate typical characteristics of gliomagenesis including increased cellularity, cellular and nuclear atypia, increased mitotic figures, pseudopalisading cells, and hypervascularity (Fig. 4A, bottom left panel). Tumors with AJAPI overexpression are significantly smaller than AJAPI negative tumors (Fig. 4A, top right panel, and Fig. 4B). Glioma xenografts that overexpressed AJAPI resulted in longer survival than AJAPI-negative cells. We evaluated a U87 xenograft with a pEGFP-N1 vector versus U87 xenograft with AJAPI overexpression via a pEGFP-AJAPI construct (Fig. 4C, log-rank P=0.01).

AJAPI loss demonstrates worse survival in GBM patients. Previously, using four independent sets of genomic data and human tumor samples, we found that the unique loss of AJAPI is a frequent event in up to 16% of GBM primary tumors (10). In this study, we obtained linked clinical data from 35 adult GBM patients with measured protein expression of AJAPI.

All patients underwent standard of care therapy, including gross total resection of visible enhancing tumor (>95% on postoperative MRI), followed by at least 6 months of concurrent radiation therapy and temozolomide. In this cohort, age <45 years predicted better survival (P<0.05), but age was not correlated with AJAPI expression (P>0.1). Patients with markedly reduced or absent AJAPI expression clearly trended toward worse survival than patients with intermediate or normal AJAPI expression (Fig. 4D). This is consistent with our prior observation of the public database Rembrandt, where all glioma patients were assessed (10). Those with downregulation of AJAPI expression (>2-fold loss, n=206) clearly had significantly worse survival than those with intermediate or normal expression (n=91; P=0.0056) (10).

Discussion

Despite surgical removal of the visible tumor, GBM universally recurs because of the high migratory potential of tumor

cells into nearby brain parenchyma (3). Determining how to effectively target critical signaling pathways or mediators of specific tumor cell features such as migration is of paramount importance for treating this disease.

In our prior study, we used digital karyotyping, microarrays and the TCGA dataset to investigate chromosome 1p36, a hotspot for genetic loss in cancers (10). With high-resolution mapping, we and others have demonstrated the frequent loss of AJAPI expression in GBM primary tumors (10,11). Others have demonstrated the loss of expression of AJAPI in neuroblastomas (14), oligodendrogliomas (13), and ependymomas (21). We discovered genomic deletion of AJAPI in up to 16% of these tumors and cell lines; however, a much larger percentage (86-92%) had loss of expression of this gene.

Epigenetic silencing via cytosine methylation is a well-established and extensively used mechanism for gene regulation in numerous cancers, including GBM. Using mutational and methylation analyses, we previously showed that lack of AJAPI expression is not due to promoter or gene mutation but is epigenetically silenced by promoter hypermethylation (10). Cogdell *et al* recently demonstrated increased methylation that correlated with less AJAPI expression in oligodendroglioma (22). In our large series of primary GBM tumors and cell lines, 65 and 90%, respectively, showed widespread evidence of AJAPI methylation. Targeting methylated genes in GBM may be a viable option since demethylating agents have shown efficacy in preclinical studies (10) and clinical trials.

In this study, we have further explored the expression of AJAPI in a large panel of glioma cells to identify cells with absent or low expression. After establishing glioma cells that stably overexpress AJAPI on the plasma membrane, we provide ample evidence that modulating gene expression clearly affects tumor cell invasion and migration. Loss of AJAPI resulted in increased invasion and migration. We were able to reverse the phenotype by knocking down the gene in cells that overexpress it. In addition, AJAPI expression clearly affects cellular morphology. Glioma cells with the loss of AJAPI took on a more amoeboid shape with extensive extensions, which suggests that these cells were actively exploring their environment to find a more suitable place to which to migrate and proliferate. In our xenograft tumor models, overexpression of AJAPI markedly slowed tumor growth and increased survival. Finally, we demonstrate a clear association of the loss of AJAPI expression and worse survival in our cohort of GBM patients, consistent with data from the publicly available data set Rembrandt (10,22). These observations and hypotheses regarding the role of AJAPI in GBM warrant further investigation.

Little is known about the interactions of AJAPI except in the context of epithelial cells. In polarized epithelial cells, AJAPI is a transmembrane protein that interacts with E-cadherin- β -catenin complexes (12), the adaptor protein complex AP-1B (20), and CD147 (16). These recent findings suggest a potential role for AJAPI in cell-cell and cell-ECM interactions that could be involved in cell motility, migration, and invasion. As in epithelial cells and several cancers, E-cadherin and β -catenin may play a critical role in GBM cell migration as well. Modulation of the cadherin/catenin system may be facilitated by AJAPI in GBM; however,

whether and how this system interacts with AJAPI is unknown.

Paradoxically, whereas the loss of AJAPI expression in HeLa cells results in decreased invasiveness (16), its overexpression in glioma cells results in decreased invasiveness (13). McDonald *et al* also found in U251 tumor cells that AJAPI overexpression decreased cell adhesion on ECM components and decreased migration in wound-healing assays (13), consistent with our results in stable transfections. These studies emphasize that AJAPI may serve very different roles in different scenarios. Given these findings and our evidence of frequent loss of AJAPI expression in GBM, we hypothesize that this phenomenon contributes to tumor cell migration in GBM as well.

During invasive migration, many cancer cells use secreted and surface-localized factors, such as matrix metalloproteinases, serine proteases, and cathepsins to proteolytically clear and remove different types of ECM substrates at their interface, including collagens, laminins, vitronectin, and fibronectin. Many of these processes appear relevant to glioma cell migration as well (23). Additionally, the mechanisms required for GBM cell adhesion likely differ from those needed for active GBM migration (24). Glioma cell migration and invasion is a complex combination of multiple molecular processes, including altered tumor cell adhesion to a modified ECM, secretion of proteases, and modified actin cytoskeleton (24). Delineating the role of these processes in GBM migration and interaction with AJAPI will require further investigation.

Other investigators have seen *AJAPI* deletion in brain tumors. In oligodendroglioma, McDonald *et al* found the specific deletion of *AJAPI* in 6 of 177 (3.4%) tumors (13), and Dong *et al* found its deletion in 6 of 52 (12%) (25). The loss of loci on the distal arm of chromosome 1p is more frequently seen in oligodendroglioma than in GBM, where the combined loss of 1p and 19q is predictive of prolonged survival (26). White *et al* defined a single region within 1p36.3 that was consistently deleted in 25% of neuroblastomas, an extracranial tumor of the sympathetic nervous system commonly seen in children (27). While mapping the smallest region of a consistently deleted segment at 1p36.31 in neuroblastomas, Fujita *et al* found 23 genes, including *AJAPI* (28). Milde *et al* showed the loss of *AJAPI* in a progressively metastasizing ependymoma, another type of brain glioma (21). Our work here demonstrates that the loss of 1p as seen in numerous studies by us and others may be most significant because of the subsequent loss of AJAPI on 1p. The loss of AJAPI expression may be the critical alteration in this oncogenomic chromosomal 'hotspot' for glioblastoma and other cancers.

In this study, we extend our discovery of the frequent loss of expression of AJAPI in GBM by demonstrating its clear role in tumor cell invasion and migration. Expression of AJAPI in tumors results in slower tumor growth, increased survival in animal models, and correlates with survival in GBM patients. AJAPI represents an exciting therapeutic target and prognostic factor in GBM.

Effective reduction in the extensive migration of GBM cells could greatly complement surgical resection of the primary tumor focus in this universally fatal brain tumor. Studies have already shown that glioma invasion can be the target of

directed therapies and that these approaches may augment the efficacy of traditional therapies (29).

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