Deletion or Epigenetic Silencing of AJAP1 on 1p36 in Glioblastoma

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Abstract
Glioblastoma is universally fatal because of its propensity for rapid recurrence due to highly migratory tumor cells. Unraveling the genomic complexity that underlies this migratory characteristic could provide therapeutic targets that would greatly complement current surgical therapy. Using multiple high-resolution genomic screening methods, we identified a single locus, adherens junctional associated protein 1 (AJAP1) on chromosome 1p36 that is lost or epigenetically silenced in many glioblastomas. We found AJAP1 expression absent or reduced in 86% and 100% of primary glioblastoma tumors and cell lines, respectively, and the loss of expression correlates with AJAP1 methylation. Restoration of AJAP1 gene expression by transfection or demethylation agents results in decreased tumor cell migration in glioblastoma cell lines. This work shows the significant loss of expression of AJAP1 in glioblastoma and provides evidence of its role in the highly migratory characteristic of these tumors. Mol Cancer Res; 10(2); 208–17. © 2012 AACR.

Introduction
Glioblastoma is one of the most common and most malignant primary brain tumors in adults (1). Successful resection of more than 98% of the tumor alone provides about 8 months of survival because tumor cells have invariably begun migrating away from the visible primary tumor focus. Adjuvant chemo- and radiotherapies provide some benefit. In a recent meta-analysis, an increase in median survival from 12.1 to 14.6 months was observed in glioblastoma patients after multimodal therapy with gross total resection, radiotherapy, and chemotherapy with the alkylating drug temozolomide (2).

To make significant strides in glioblastoma treatment, novel insights into the biology of these tumors are required. Recent genome-wide studies continue to elucidate the common genetic changes and altered signaling cascades in glioblastoma (3, 4). These studies also highlight the large number of genetic changes due to the acquired mutator phenotype that may not play a central role in tumor initiation, proliferation, or cell migration (5). Despite the plethora of genetic alterations seen in glioblastoma, most revealing genetic studies have typically implicated only a few altered signaling pathways, namely, activating events in the PTEN or receptor tyrosine kinase pathways and decreased activity in the RB1 or TP53 signaling cascades (3, 6).

For many cancers the distal arm of chromosome 1p has been a mutational hotspot. A variety of human cancers, including glioblastoma, neuroblastoma, oligodendroglioma, leukemia, lymphoma, squamous cell carcinoma, breast cancer, and prostate cancer frequently have genetic deletions at 1p36. In glioblastoma, methylation seems to be a common event that also silences gene expression of tumor suppressor genes, including RB1, p16INK4a, p14ARF, MGMT, TIMP3 (7), TMS1/ASC (8, 9), CASP8 (10), RUNX3, and RES (11). Unfortunately, the vast majority of these genetic and epigenetic events have yet to provide meaningful therapeutic or prognostic markers, except possibly for the case of MGMT (12).

One unfortunate characteristic of glioblastoma is its robust propensity to migrate from the primary focus of tumor initiation to distant sites and to recur despite aggressive local and system therapies. Effective therapies that could impede this process of tumor cell migration could provide potentially curative adjunctive therapy to the current standard of care. Adherens junctional associated protein-1 (AJAP1; also known as Shrew1) has recently been discovered as a novel transmembrane component of adherent junctions in epithelial cells (13).

Materials and Methods

Tumor samples
Tumor samples (>95% pure tumor) were obtained from the Duke Preston Robert Tisch Brain Tumor Center Tissue
Biorepository, in accordance with an Institutional Review Board–approved protocol. 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.

Digital karyotyping
Digital karyotyping libraries were constructed as previously described (14). Eighteen glioblastoma libraries (14 adult and 4 pediatric) were generated and the data combined with 9 glioblastoma libraries from The Cancer Genome Atlas (TCGA) database, for a total of 27 libraries. Digital karyotyping protocols and software for extraction and analysis of genomic tags are available at http://www.digitalkaryotyping.org/index.html.

The Cancer Genome Atlas
Using the TCGA Data Portal at http://tcga-data.nci.nih.gov/tcga/findArchives.htm, we downloaded all copy number data generated at the Memorial Sloan Kettering Cancer Center. All normal blood, normal tissue, cell line, and duplicate data were removed yielding 221 glioblastoma primary tumors for analysis. TCGA raw data was reformatted for analysis by Nexus Copy Number Professional software (Biodiscovery, Inc.).

REMBRANDT
Data with regard to expression, copy number, and survival were obtained from http://tr.ncbi.nlm.nih.gov/rembrandt website, release date 11-13-09. This dataset included 506 samples for gene expression data, 722 for copy number data, and 891 for clinical data.

Illumina’s HumanHap 550 Quad and 610 Duo Genotyping BeadChips
The Illumina BeadChips enable whole-genome genotyping of more than 555,000 and 610,000 tag single-nucleotide polymorphism (SNP) markers, respectively, derived from the International HapMap Project (www.hapmap.org). Thirty-five tumors were analyzed by the 550 chip, and another 43 tumors were analyzed by the 610 chip for a total of 78 unique samples. Blood and normal cortex were included. Genomic DNA was isolated with the QIAamp DNA Mini Kit (Qiagen), hybridized to the BeadChip microarrays, and data analyzed by using Illumina BeadStudio software. Illumina raw data was reformatted by Nexus.

Serial analysis of gene expression
Serial analysis of gene expression (SAGE) data were reviewed on the NCBI Cancer Genome Anatomy Project repository (http://cgap.nci.nih.gov/SAGE). In addition, we identified the SAGE sequence tags for the AJAP1 locus previously published (Supplementary Table S8 in Parsons and colleagues; ref. 15).

RNA extraction, cDNA synthesis, quantitative PCR
Total RNA was isolated by using RNeasy Mini Kit (Qiagen) and reverse transcribed by using an iScript cDNA Synthesis Kit (Bio-Rad). cDNA amplification was monitored by using a SYBR Green protocol (Sigma) on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Forward and reverse primers were designed at different exons. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as control. 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 AJAP1 gene was normalized to that of GAPDH. As a reference, we used adult human brain RNA (BioChain Institute).

Bisulfite treatment of DNA and methylation-specific quantitative PCR
Genomic DNA was isolated from fresh-frozen tissue samples and cell lines using Wizard Genomic DNA Purification Kit (Promega). Using Methprimer (http://urogene.org/methprimer), we carried out a CpG island search throughout intragenic and promoter regions up to 3,000 bp upstream of the start site for AJAP1. Bisulfite modification of genomic DNA was done using EZ DNA Methylation-Gold Kit (Zymo). Methylation-specific quantitative PCR (qPCR) was carried out with 2 μL of bisulfite-treated DNA using a SYBR Green PCR Kit (Qiagen) on an ABI PRISM 7900 HT system (Applied Biosystems) according to the manufacturer’s instructions. Two sets of methylation-specific primers and 2 sets of unmethylation-specific primers were used. The percentage of methylation in each tumor specimen was calculated by the following equation: % meth = 100/[1 + 2ΔC(t)meth−unmeth]). ΔC = C(t)meth−unmeth was calculated by subtracting the Ct values of methylated AJAP1 signal from the Ct values of unmethylated AJAP1 signal. Each sample was run in duplicates for analysis.

Cell culture and drug treatment
Cell lines were maintained in Improved MEM Zinc Option Medium (Invitrogen) supplemented with 10% FBS at 37°C, 5% CO2. For demethylation studies, glioma cells were seeded in 6-well plates, allowed to attach overnight, and treated with 5 μmol/L 5-aza-2'-deoxycytidine (AZA; Sigma) for 4 days, followed by a 24-hour treatment with 0.1 μmol/L trichostatin A (TSA; Sigma). In control groups, the same concentration of dimethyl sulfoxide was used to treat cells. Fresh drug and medium were changed every 24 hours.

AJAP1 expression plasmids
The AJAP1 cDNA was isolated from normal cortex by a PCR and subcloned into the pEGFP-N1 expression plasmid. The pAJAP1-EGFP expression plasmid was transfected with Lipfectamine 2000 (Invitrogen). The expression of AJAP1 was confirmed by fluorescence microscope and Western blot (Supplementary Fig. S6).

AJAP1 knockdown studies
Two different siRNA oligonucleotides for AJAP1 inhibition (AJAP1 Stealth RNAi siRNA HSS148198 and AJAP1 Stealth RNAi siRNA HSS148199) and scrambled siRNA
for negative control were obtained (Invitrogen). A total of $2 \times 10^5$ GBM cells were seeded in 6-well plates and grown to 90% confluence in 1.5 mL culture medium. After treatment, cells were then washed with PBS and transfected by Lipofectamine 2000 (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 48 hours and then added to migration assay as described below. RNA knockdown was determined by cDNA synthesis as previously described and visualization on agarose gel. At 48 hours, the loss of AJAP1 was optimal, so this time was selected for the procedure and applied in all knockdown experiments.

**Cell proliferation assay**

Cells were plated at $1 \times 10^5$ cells per 6-well plate in triplicate for each cell line. Cell viability was determined by the MTT assay.

**Transwell migration assay**

Transwell cell migration assays were done by using modified Boyden chambers (Transwell). In brief, cells were trypsinized, washed, and suspended in serum-free medium at $10^6$ cells/mL. Hundred microliters of cells were placed into the upper chambers and allowed to migrate for various time points toward the bottom well with 10% FBS. Cells that migrated to the underside of the chamber were fixed with 3.7% paraformaldehyde, stained with 0.5% crystal violet, and the membranes were dissolved with 33% acetic acid. Cells were photographed and hand-counted by light microscopy, as well as optically densitized at 595 nm by an ELISA plate reader.

**Statistical analysis**

All experiments were done in triplicate. Statistical significance was set a priori at $P \leq 0.05$. Analyses were done with Microsoft Excel and SAS E-guide statistical packages (SAS, Inc.).

**Results**

**AJAP1 is deleted in glioblastoma**

We used different methods to carry out genome-wide screens in glioblastoma samples for genetic alterations. We initially used digital karyotyping, which can be reliably used to identify chromosomal changes, amplifications, deletions, and the presence of foreign DNA sequences (14, 16). We evaluated 27 glioblastoma libraries with an average of 175,000 genomic tags per library, permitting analyses of loci distributed at an average distance of 30 kb throughout the genome. We also carried out a genome-wide search for alterations in 78 different glioblastoma primary tumors using the Illumina HumanHap 550 Quad and 610 Duo BeadChip microarrays.

Maps of digital karyotyping libraries and Illumina microarrays all revealed widespread subchromosomal changes in glioblastoma (Fig. 1A and B). Losses were more frequent than gains. High-resolution mapping revealed numerous known and unknown genetic alterations in glioblastoma tumors. For example, amplification of \textit{EGFR} on chromosome 7, loss of \textit{CDKN2A} on chromosome 9, and chromosome 10q LOH were clearly shown in our digital karyotyping libraries (Supplementary Fig. S1) and Illumina data (Fig. 1B). These findings served as important internal positive controls.
One genetic loss was located at chromosome 1:3,812,102–5,418,455 on the distal arm of chromosome 1p36 in 4 of our 27 digital karyotyping libraries (15%; Fig. 2A). Examination of the known public human genome database identified one known gene in this segment, AJAP1 (Fig. 2A). These AJAP1 genomic losses consisted of 3 homozygous and 1 LOH deletions. Using Illumina Human-Hap BeadChip SNP microarrays, we examined 78 glioblastoma samples and discovered 3 LOH deletions of AJAP1 in the total sample (4%) of tumors (Fig. 2B). To validate these findings, we conducted qPCR on our original set of 80 primary glioblastoma tumors and found gene deletion in 15% (7 of 12 LOH and 5 of 12 homozygous deletions; Supplementary Table ST1). In summary, our analysis of this hotspot for genetic alterations on chromosome 1p36 in 105 samples using independent sets of genomic data revealed the unique deletion of AJAP1 in up to 16% of glioblastoma tumors.

AJAP1 expression is downregulated in glioblastoma tumors and cell lines

We initially examined AJAP1 expression in 13 primary glioblastoma samples, 8 glioma cell lines, and 4 normal brain samples by using qPCR. We found that AJAP1 expression was markedly reduced or absent in 92% (12 of 13) primary glioblastomas (Fig. 3A, TB samples) and all glioblastoma cell lines tested (Fig. 3A, D and U samples). We expanded this study to our entire original set of 80 primary tumors and found reduced or absent expression in 86% (Supplementary Table ST1). We also conducted a search of a public cancer genome SAGE database (http://cgap.nci.nih.gov/SAGE) and confirmed that AJAP1 expression is significantly reduced in glioblastoma, with an average of 3 AJAP1 SAGE tags in glioblastoma compared with 12 tags in normal human brain. Finally, we explored the sequence tag density for AJAP1 in another SAGE database previously published (see Supplementary Table S8 in Parsons and colleagues; ref. 15). In this database of 16 glioblastoma tumors, 14 tumors (88%) had sequence tag densities markedly reduced when compared with a normal sample (Fig. 3B).

Furthermore, we explored the REMBRANDT public database and found intermediate or low AJAP1 expression in all 196 glioblastoma samples when compared with normal tissue, consistent with the 86% to 92% of our primary glioblastoma tumors with reduced or absent...
expression. When compared with all gliomas (Supplementary Fig. S3, blue line) in the database, those with down-regulation of AJAP1 expression (green line, >2.0-fold loss, \(n = 206\)) clearly have a significantly worse survival than those with intermediate expression (yellow line, \(n = 91\); \(P = 0.0056\)).

AJAP1 loss of expression is due to promoter methylation

Through our genome-wide screens, we discovered the frequent deletion of AJAP1 in glioblastoma. Expression studies revealed that loss of AJAP1 gene expression seemed to be much more common than gene deletion. Expression was reduced or absent in 86% to 92% of primary glioblastoma tumors and all glioma cell lines, whereas the gene was deleted in up to 16% of the tumor samples. These results suggested other mechanisms of loss of gene expression. We carried out an exon-by-exon analysis of our original 80 glioblastoma tumors and glioma cell lines, and no point mutations were identified in any exons. We hypothesized that promoter methylation may account for AJAP1 gene silencing. An extensive search revealed 21 CpG candidate island hotspots in the genomic sequence of the AJAP1 promoter region that may serve as sites of gene silencing by methylation (Fig. 4A). Using quantitative methylation-sensitive PCR on bisulphite-treated samples, we found that the AJAP1 promoter was often methylated in glioblastoma primary tumors and glioma cell lines (Fig. 4B–C). We initially observed significant AJAP1 promoter methylation in 13 of 20 primary glioblastoma (65%) and 9 of 10 cell lines (90%; Fig. 4B). Normal brain samples were found to be unmethylated (Fig. 4B). Glioblastoma cell lines U87MG and D54MG showed the lowest levels of gene expression (Fig. 3A) and the highest number of CpG islands to be methylated (Fig. 4B). We then examined our entire set of 80 primary tumors and found significant methylation (i.e., >10% of CpG islands tested) in 63% (Supplementary Table ST1). We found a clear correlation of loss of expression and the presence of methylation (Supplementary Fig. S4, A and B).
Spearman’s rank correlation, $R = -0.7$, $P < 0.005$) of $AJAP1$. Hundred percent of tumors with normal expression, 50% with intermediate expression, and 26% with low/absent expression had no methylation.

**Loss of $AJAP1$ expression is associated with increased glioma cell proliferation and migration**

Prior studies suggest a potential role for $AJAP1$ in cell–cell and cell–extracellular matrix interactions that could be
involved in cell motility, migration, and invasion (13, 17–19). These studies indicated that the effect of AJAP1 on tumor cell migration may depend upon the specific tumor type and its environment. On the basis of these findings and our evidence of loss of expression in glioblastoma, we hypothesize that AJAP1 may contribute to glioblastoma cell migration. We chose D409MG, a glioblastoma cell line with very low AJAP1 expression (Fig. 3A) and evidence of promoter methylation (Fig. 4B). Overexpression of AJAP1 in D409MG with the pEGFP-AJAP1 expression resulted in a marked decrease in cell proliferation (Fig. 5A) and migration through Transwell migration assays (Fig. 5B) as compared with the same cell type transfected with the empty vector pEGFP-N1 (Fig. 5B). We show similar findings in another glioma cell line as well (Supplementary Fig. S5).

Demethylation restores AJAP1 expression and function

Because AJAP1 can be epigenetically silenced in glioblastoma primary tumors and cell lines, pharmacologic reversal of this epigenetic silencing may be a viable option for restoring normal expression and function. To test this hypothesis, we selected the glioblastoma cell lines D54MG and U87MG, which show very low AJAP1 expression (Fig. 3A) and extensive promoter methylation (Fig. 4B). Both cell lines were exposed to the methyltransferase inhibitor AZA and the histone deacetylase inhibitor TSA. AZA and TSA have been extensively used in preclinical and clinical studies for demethylating genes (20). In D54MG and U87MG, AJAP1 expression is undetectable without exposure to either agent (Fig. 6A, lanes 1; Supplementary Fig. S2), but expression is dramatically increased upon exposure to AZA (Fig. 6A, lanes 2; Supplementary Fig. S2) or TSA (Fig. 6A, lanes 3; Supplementary Fig. S2). Densitometric quantification of the PCR products showed that AJAP1 expression in both cells increased more than 50-fold (Fig. 6B). Bisulphite sequencing confirmed that these reagents dramatically reduce the number of methylated CpG islands in the AJAP1 promoter (Fig. 6C). In D54MG (similar results with U87MG), restoration of AJAP1 expression by demethylation resulted in a significant decrease in migration after treatment (Fig. 6D, hatched bar). Furthermore, we were able to partially “rescue” this phenotype and increase migration by knocking down the AJAP1 gene with siRNA after demethylation treatment (Fig. 6D, gray and diamond bars). These results confirmed that AJAP1 expression is epigenetically silenced in glioma cells, promoter methylation can be reversed pharmacologically, and restoration of AJAP1 expression by demethylation treatment decreases glioma cell migration.

Discussion

With high-resolution genome-wide mapping, we showed in this study the deletion of AJAP1 in many glioblastoma cell lines and primary tumors (Figs. 1 and 2). In a total of 105 samples, we discovered that up to 16% have AJAP1 deleted; however, a much larger percentage (86%–92%) have loss of expression (Fig. 3). Other investigators have seen AJAP1 deletion in glial brain tumors. In oligodendroglioma, McDonald and colleagues found the specific deletion of AJAP1 in 6 of 177 (3.4%) tumors (19). Dong and colleagues reported 2 contiguous minimally deleted regions on chromosome 1p36.31–p36.32 in oligodendroglial tumors, one of which contained AJAP1 as the only gene (21). The loss of loci on the distal arm of chromosome 1p is more frequently seen in oligodendroglialomas than glioblastoma, where the combined loss of 1p and 19q is frequent and predictive of prolonged survival (22). Oligodendrogliomas and glioblastomas have some differences; however, these 2 glial derived tumors may have important similarities that can be taken advantage for therapeutic design. The importance of this study on glioblastoma primary tumors and cell lines is that it greatly builds on the prior findings in oligodendroglomas.
We hypothesize that AJAP1 may indeed play a similar role in cell adhesion and migration for both of these brain tumors. White and colleagues 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 (23). AJAP1 was 1 of 6 predicted genes in this deleted region. CHD5 was recently found to be a strong tumor suppressor gene candidate deleted from 1p36.31 in neuroblastomas, and inactivation of the second allele was speculated to occur by an epigenetic mechanism (24). This was discovered while mapping the smallest region of a consistently deleted segment at 1p36.31 that contained 23 genes, including AJAP1 (24). Milde and colleagues recently showed the loss of AJAP1 in a progressively metastasizing ependymoma (25).

In polarized epithelial cells, AJAP1 is a transmembrane protein that interacts with E-cadherin–β-catenin complexes (13), the adaptor protein complex AP-1B (17), and CD147 (26). These recent findings suggest a potential role for AJAP1 in cell–cell and cell–extracellular matrix interactions that could be involved in cell motility, migration, and invasion. Little is known about the interactions of AJAP1, except in the context of epithelial cells. Modulation of the cadherin/catenin system may be facilitated by AJAP1 in glioblastoma; however, whether and how this system interacts with AJAP1 is unknown.

Paradoxically, the loss of AJAP1 expression in HeLa cells results in decreased invasiveness (26); however, its overexpression in tumor cells also results in decreased invasiveness (19). In their study that included oligodendrogliomas, McDonald and colleagues found in U251 cells (glioblastoma cell line) that AJAP1 overexpression decreased cell adhesion on extracellular matrix components and decreased migration in wound healing assays (19). These studies emphasize that AJAP1 may serve very different roles in different scenarios. On the basis of these findings and our evidence of common loss of expression in glioblastoma, we hypothesized that it may contribute to tumor cell migration in glioblastoma.

Consistent with the results of McDonald and colleagues, we also see a significant effect on tumor cell migration in glioblastoma. We have reviewed our available clinical data for the tumors tested in this article and do not see a significant difference in AJAP1 deletion, expression, or methylation between primary and secondary glioblastoma.

There is an extensive array of other factors implicated in glioma cell migration in which the potential relationship to AJAP1 expression is unexplored (4, 27). During invasive migration, cancer cells use secreted, surface-localized and...
intracellular matrix metalloproteinases, serine proteases, and cathepsins to proteolytically clear and remove different types of extracellular matrix substrates at their interface, including collagens, laminins, vitronectin, and fibronectin. Some of these processes may be relevant to glioma cell migration as well. The role of these processes in glioblastoma migration and interaction with AJAP1 remains for further investigation.

Epigenetic silencing via cytosine methylation is a well-established and extensively used mechanism for gene regulation in numerous cancers, including glioblastoma. Genome-wide screens of glioma cells treated with AZA and TSA reveal more than 160 genes upregulated by these treatments (28). Using mutational and methylation analyses, we showed that AJAP1 expression is not due to mutation but is epigenetically silenced with promoter methylation in many cases. In our large series of primary tumors and cell lines, we see widespread evidence of AJAP1 methylation (Fig 4B). Furthermore, this mechanism of gene silencing is effectively reversed with the common demethylating agents AZA and TSA (Fig 6). Restoration of AJAP1 expression by these pharmacologic agents returned its function by reducing tumor cell migration (Fig 6D). We show that demethylation agents can target AJAP1 (Fig 6D); however, because we are able to only partially recover migration by knocking down AJAP1 after demethylation treatment, we hypothesize that AZA/TSA treatment likely alters expression of other unknown genes that effect migration. Importantly, not all samples with low or no expression of AJAP1 show evidence of promoter methylation. Clearly, our data support the observation that other mechanisms, other than DNA methylation and gene mutation, also play a role in AJAP1 expression. Targeting methylated genes in glioblastoma may be a viable option as demethylating agents have shown efficacy in preclinical and clinical trials (20, 29).

Although all the methylated glioblastoma tumors showed reduced or silenced expression of AJAP1, one primary tumor (TB2479) and one cell line (D336MG) exhibited reduced AJAP1 expression in the absence of methylation in the analyzed region. It is likely that transcriptional activation of AJAP1 may also be influenced by other mechanisms, such as accessibility of AJAP1 regulator proteins or specific transcription factors.

In this study, we report that AJAP1 is deleted and epigenetically silenced in some glioblastomas. Pharmacologic demethylation treatments return expression and restore function by decreasing tumor cell migration. There is an extensive literature on factors involved in glioblastoma migration in which the relationship to AJAP1 is unexplored. This represents an exciting therapeutic target in the treatment of glioblastoma. Effective targeting of migrating glioblastoma cells through chemotherapies or radioconjugates could greatly impact survival in this deadly brain tumor and potentially eliminate the morbidities of surgical resections.

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 (30).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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