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# Tumor-associated methylation of the putative tumor suppressor *AJAP1* gene and association between decreased *AJAP1* expression and shorter survival in patients with glioma

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**Abstract**

Allelic loss of the short arm of chromosome 1 has been observed frequently in a wide spectrum of cancers, most frequently in oligodendroglioma. In our previous studies, we evaluated 177 oligodendroglial tumor samples and identified the *AJAP1* gene (formerly *Shrew1*) in the consensus region of deletion. AJAP1 is a transmembrane protein found in adheren junctions and functions to inhibit glioma cell adhesion and migration. Whereas a putative tumor suppressor gene, we did not detect *AJAP1* gene mutations. In subsequent studies, we found that *AJAP1* was underexpressed in oligodendrogliomas relative to normal brain tissues. Bioinformatic analysis revealed the presence of CpG islands in the promoter of *AJAP1*. Methylation analysis of the *AJAP1* promoter identified hypermethylation in 21% of oligodendrogliomas ($n$ = 27), and the degree of methylation correlated with low levels of *AJAP1* expression ($P$ = 0.045). The *AJAP1* promoter was also highly methylated in a wide spectrum of cell lines ($n$ = 22), including cell lines of glioblastoma. Analysis of the National Cancer Institute's REMBRANDT dataset, which contains 343 glioma samples, indicated that low *AJAP1* gene expression was associated with decreased survival. Thus, both genetic (gene deletion) and epigenetic alterations (promoter methylation) are likely mechanisms that inactivate the putative tumor suppressor *AJAP1* in gliomas, which contributes to poor prognosis.

**Key words** Methylation, epigenetics, *AJAP1*(*Shrew1*), *1p36*, glioblastoma, The Cancer Genome Atlas, survival

Cancer is caused by an accumulation of genetic and epigenetic alterations. Gene silencing by hypermethylation of CpG islands in promoter regions is a common epigenetic abnormality in cancer and may lead to a loss of function of tumor suppressor genes, such as *p16, MGMT, RASSF1A,* and *hMLH1*[1-4].

Oligodendroglioma is one of the most common primary neoplasms of the central nervous system among adults in the fourth and fifth decades of life[5]. A common and characteristic chromosomal abnormalities observed in oligodendroglioma is allelic loss of 1p [6-11]. Earlier deletion mapping studies identified most consensus deletion regions involving the 1p36 region[8,11,12]. Additional mapping studies in recent years have further narrowed the consensus regions of deletion. One study with 52 oligodendrogliomas identified two regions of interest: one region contained the *DFFB* gene, which we also found to be down-regulated in oligodendrogliomas with the 1p/19q deletion [13], whereas the other region contained the *AJAP1* gene [14]. In our earlier studies with 177 oligodendrogliomas, we found that the second region, containing *AJAP1*, was in the consensus region of deletion [15]. *AJAP1* is a putative tumor suppressor gene found on chromosome 1 in the 1p36 region commonly associated with cancer [11] and has been implicated in

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cancer cell invasion, adhesion, and migration [15,16]. Although we did not find mutation of the *AJAP1* gene in oligodendrogliomas, its expression at transcript level was lower than in normal brain tissue. Immunohistochemistry using clinical samples revealed an association between *AJAP1* levels and survival in patients with astrocytic glioma [17]. Additionally, restoration of *AJAP1* expression in glioma cell line inhibited adhesion and migration [15]. Thus, *AJAP1* may be a tumor suppressor whose function can be attenuated by a loss in copy number and a decrease in expression.

In this study, we investigated the possible contribution of promoter methylation to decreased *AJAP1* expression in cancer cells. Analysis of an independent microarray dataset, the Repository for Molecular Brain Neoplasia Data (REMBRANDT) [18] linked the *AJAP1* expressions levels to survival.

## Materials and Methods

### Samples and DNA isolation

The records of 37 patients who underwent treatment for oligodendroglial tumors at The University of Texas MD Anderson Cancer Center between 1981 and 2002 were collected and reviewed with approval from the Institutional Review Board. These tumors were initially diagnosed by neuropathologists at MD Anderson as 1) low-grade oligodendroglioma or mixed oligoastrocytoma, 2) anaplastic oligodendroglioma (AO) or mixed oligoastrocytoma, or 3) glioblastoma with a significant oligodendroglial component, and the diagnoses were later confirmed by two of the authors (K.A. and G.F.). Mixed tumors were included in this study because clear pathologic discrimination between the diffuse glioma subtypes is often difficult and subjective and, as a group, oligoastrocytomas often exhibit the 1p/19q deletion, a genetic signature that has been observed in both the oligodendroglial and astrocytic components of mixed tumors[19].

Tissue for DNA isolation was obtained from paraffin-embedded samples. Each tissue block was histologically assessed for tumor by a neuropathologist (K.A.). Sections were directly cut from the block for DNA isolation if at least 90% of the tissue was determined to be tumor. If the proportion of tumor was < 90%, 10 to 20 unstained slides were prepared from the block, and tumor tissue was dissected from normal tissue. DNA was isolated by digesting deparaffinized tumor sections for 3 to 5 days with proteinase K at 55℃ [0.5 mg/mL in 100 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 8.0), 25 mmol/L ethylenediaminetetraacetic acid, 0.5% sodium dodecyl sulfate], followed by a phenol:chloroform: isoamyl alcohol extraction and isopropanol precipitation. Tissue for RNA isolation was obtained from fresh/frozen samples. Each frozen section was histologically assessed for tumor by a neuropathologist (G.F. or K.A.) and used only if at least 90% of the tissue was determined to be tumor. For RNA isolation, up to 50 mg of tissue was frozen in liquid nitrogen, crushed into powder using a mortar and pestle, and dissolved in 1 mL of Trizol reagent (Invitrogen). Then, 200 μL of chloroform was added to the sample, and the sample was vortexed at high speed for 15 s and centrifuged at 12 000 × *g* for 15 min at 4℃. After transfer of the aqueous phase to a fresh 1.5 mL Eppendorf tube, an equal volume of 70% ethanol was added, and the mixture was mixed by tube inversion. The sample was then loaded onto a Qiagen RNeasy column and centrifuged at 16 000 × *g* for 20 s. The column was washed twice with 500 μL of RPE buffer (Qiagen). RNA was eluated off the column in 50 μL of nuclease-free water. RNA was quantified using a spectrophotometer (Nanodrop) and qualified using a 2100 BioAnalyzer (Agilent). Cell lines used in the study were CaCO2, CaMa1, HL60, 468, K562, SKHep, Hep3B, HepG2, DU145, PC3, LNCaP, UC6, UC13, SVHVC, LOVO, SW480, RKO, SW480, RS4;11, Raji, MCF7, and BT474.

### Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay for *AJAP1* expression

Initial experiments were performed to determine the valid range of RNA concentrations and to demonstrate the similarity of PCR efficiencies for *AJAP1* compared to the endogenous control gene *cyclophilin*. To determine changes in *AJAP1* expression, 1 μg of total RNA from samples was reverse transcribed in a 20 μL reaction. To the RNA, 0.4 μg of random hexamers was added, and the mixture was heated at 70℃ for 10 min. The tubes were then incubated at room temperature for 10 min, and the following components were added: 1 × Superscript II RT buffer (Invitrogen), 10 mmol/L dithiothreitol (Invitrogen), 0.5 mmol/L deoxynucleotide triphosphate (ISC Bioexpress), 20 U of RNase inhibitor (Ambion), and 200 U of Superscript II RT (Invitrogen). The reaction was again incubated for 10 min at room temperature to allow primer annealing and then held incubated at 37℃ for 1 h. The reaction was spiked with an additional 200 U of Superscript II reverse transcriptase (Invitrogen) and then incubated at 42℃ for 90 min and at 50℃ for 30 min. Real-time PCR was performed on an ABI Prism 7700 using Assay-on-Demand for *AJAP1* (Hs00218945_m1) and cylophilin Vic-labeled pre-developed assay reagent (4326316E) (Applied Biosystems). A 50 μL final reaction volume containing 1 × TaqMan Universal PCR Master

Mix (Applied Biosystems) and 1 × Assay-on-Demand (Applied Biosystems) was used to amplify 50 ng of cDNA under the following cycling conditions: 10 min at 95℃ followed by 40 cycles of 95℃ for 15 s and 60℃ for 1 min. The △△Ct method was used to calculate fold-change in *AJAP1* expression in 1p loss cases relative to matched 1p intact cases. Calculations were also done to compare *AJAP1* expression in tumors with *AJAP1* expression in normal brain tissue RNA (Clontech).

### Methylation assay for the *AJAP1* promoter

Bisulfite treatment of DNA was performed as previously described [15]. We used combined bisulfite restriction analysis (COBRA) as a quantitative test to study *AJAP1* methylation in all cases[20,21]. Two separate regions in the promoter were examined and gave similar results: primers 5' - GTGTTATTYGGTTATTTTGGTAATA-3' (sense) and 5' - ACCTCCTACAACCCCCTTC-3' (antisense) were used to amplify a 134-bp fragment upstream of the promoter CpG island, whereas primers 5'-GAGTTTTYGGGTAATTTGAGTG-3' (sense) and 5'-A-CTCTTACCTCCAACCRAAAC-3' (antisense) were used to amplify a 162-bp fragment around the transcription start site. To determine the methylation status of these regions, we restricted the 134-bp fragments with *Bst*U I and restricted the 162-bp fragments with *Taq* I and *Dpn* II. All restriction products were visualized using 6% polyacrylamide gel electrophoresis followed by staining with ethidium bromide and imaging/quantitation using a BioRad Beldoc 2000 imager (BioRad). The identities of the amplified fragments were verified using digestion with multiple restriction enzymes as well as sequencing. Setup of the PCR assays included positive and negative controls, mixing experiments to rule out bias, and repeat experiments to assess reproducibility. In this analysis, these enzymes digested only methylated alleles. In selected cases, methylation of CpG sites within *AJAP*1 was confirmed using bisulfite sequencing. The 162-bp PCR products were cloned into a TA cloning vector pCR4-TOPO (Invitrogen), and plasmid clones were extracted with a QIAprep Spin Miniprep kit (Qiagen) and sequenced at the MD Anderson Core Sequencing Facility.

### Bioinformatic analysis

A Kaplan-Meier plot for gene expression data was generated using the "simple search" function within the REMBRANDT access Web site. *AJAP*1 was queried against all Affymetrix glioma data using the default up-regulation and down-regulation parameter of 2-fold. The *AJAP*1 down-regulated ($n$ = 245 cases) and intermediately regulated ($n$ = 98 cases) yielded a log-rank $P$ value of 0.001. *AJAP1* expression and methylation data were obtained from The Cancer Genome Atlas (TCGA) public database (http://www.cancergenome.nih.gov/dataportal) as available in December 2011[18]. Correlation between gene expression and methylation was assessed in terms of Pearson correlation, and $P$ value as determined using a two-tailed $t$-test.

## Results and Discussion

Although our previous sequence analysis did not reveal mutations in the coding region of the *AJAP1* (*Shrew1*) gene and noted the weak relationship between *AJAP1* gene expression and loss of heterozygosity[15], we next examined tumor tissues for epigenetic alterations affecting *AJAP1*. We theorized that a potential mechanism for the observed decreased *AJAP1* expression could be promoter methylation, not only because the *AJAP1* promoter contains a CpG island (Figure 1A) but also because promoter methylation has been shown to affect the expression of several genes in oligodendroglioma [22-25]. Thus, we theorized that *AJAP1* follows the model of the *p16* tumor suppressor gene, which has demonstrated a low rate of mutation in some tumor types but often shows attenuated expression due to promoter methylation[4].

To determine if *AJAP1* fits the *p16* model, we compared methylation using bisulfite/restriction analysis of the promoter region in 27 oligodendroglioma samples (Figure 1B, Figure 2). In our dataset, as there were no cases with methylation levels between 16% and 20%, any value in that interval was a natural choice for the threshold. Using this threshold, we detected hypermethylation in 6 (21%) of the 27 cases (Figure 3A). Of interest, 4 of the 6 cases with hypermethylation had 1p allelic loss. DNA methylation of the *AJAP1* CpG island was frequently found in cell lines from various tissue types (19 of 22 examined) and was rare in normal tissues. DNA methylation at the proximal promoter region (162-bp) of the *AJAP1* gene was confirmed in selected cases via direct bisulfite sequencing (Figure 1C).

To test the hypothesis that a high average methylation ratio was associated with decreased *AJAP1* expression, we split the cases into two groups corresponding to high and low methylation. Additionally, we subjected the methylation data to an unpaired $t$-test with two clusters, which resulted in a $P$ value < 0.05 (Figure 3A). To further verify this correlation, we obtained *AJAP1* gene expression and methylation data of 253 patients from the Cancer Genome Atlas database. The data showed two methylation sites located at chr1:4613868 and chr1:4615380, named M-4613868 and M-4615380. Distribution of gene

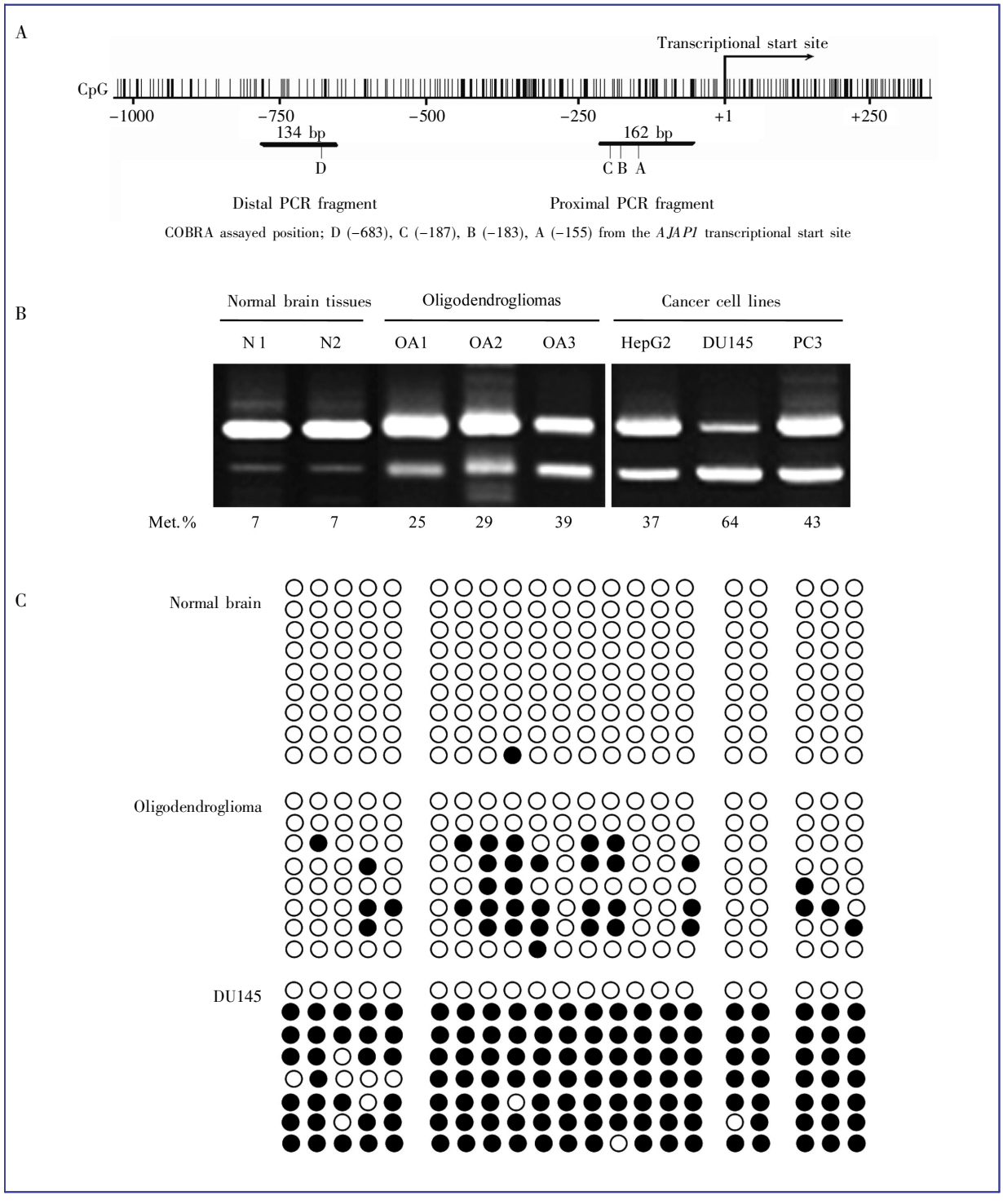


Figure 1. CpG map of the *AJAP1* promoter, COBRA and direct bisulfate sequencing for DNA methylation analysis. A, each vertical bar represents a single CpG site, and the high density of CpG sites indicates the presence of a CpG island. The transcription start site is indicated by an arrow. The 162-bp proximal PCR fragment and distal 134-bp fragment are shown with the methylation-sensitive restriction enzyme sites listed. B, an example of COBRA assay at 183-bp upstream from the transcriptional start site. The PCR product was digested with *Dpn* II that distinguished methylated DNA from unmethylated DNA after bisulfite treatment for COBRA assay. Oligodendrogliomas (OA1, OA2, and OA3) and cancer cell lines (HepG2, DU145, and PC3) had higher levels of DNA methylation than normal brain tissues (N1 and N2). C, direct bisulfite sequencing results of normal brain (N1), oligodendroglioma (OA3), and cancer cell line DU145 of proximal 162-bp PCR fragment. Black circles, methylated CpG sites; open circles, unmethylated CpG sites.

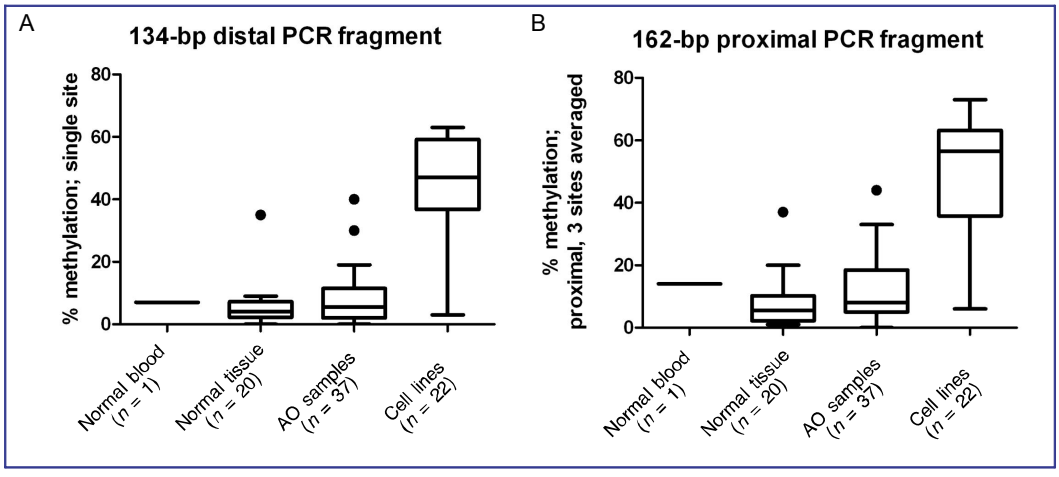


Figure 2. *AJAP1* methylation of 1 sample of normal blood, 20 samples of normal tissue, 37 samples of anaplastic oligodendroglioma (AO), and 22 cell lines. The COBRA data shown with Tukey whiskers summarizing the methylation sensitive restriction enzyme data on PCR fragments in various sample types. A, distal PCR fragment. B, proximal PCR fragment.

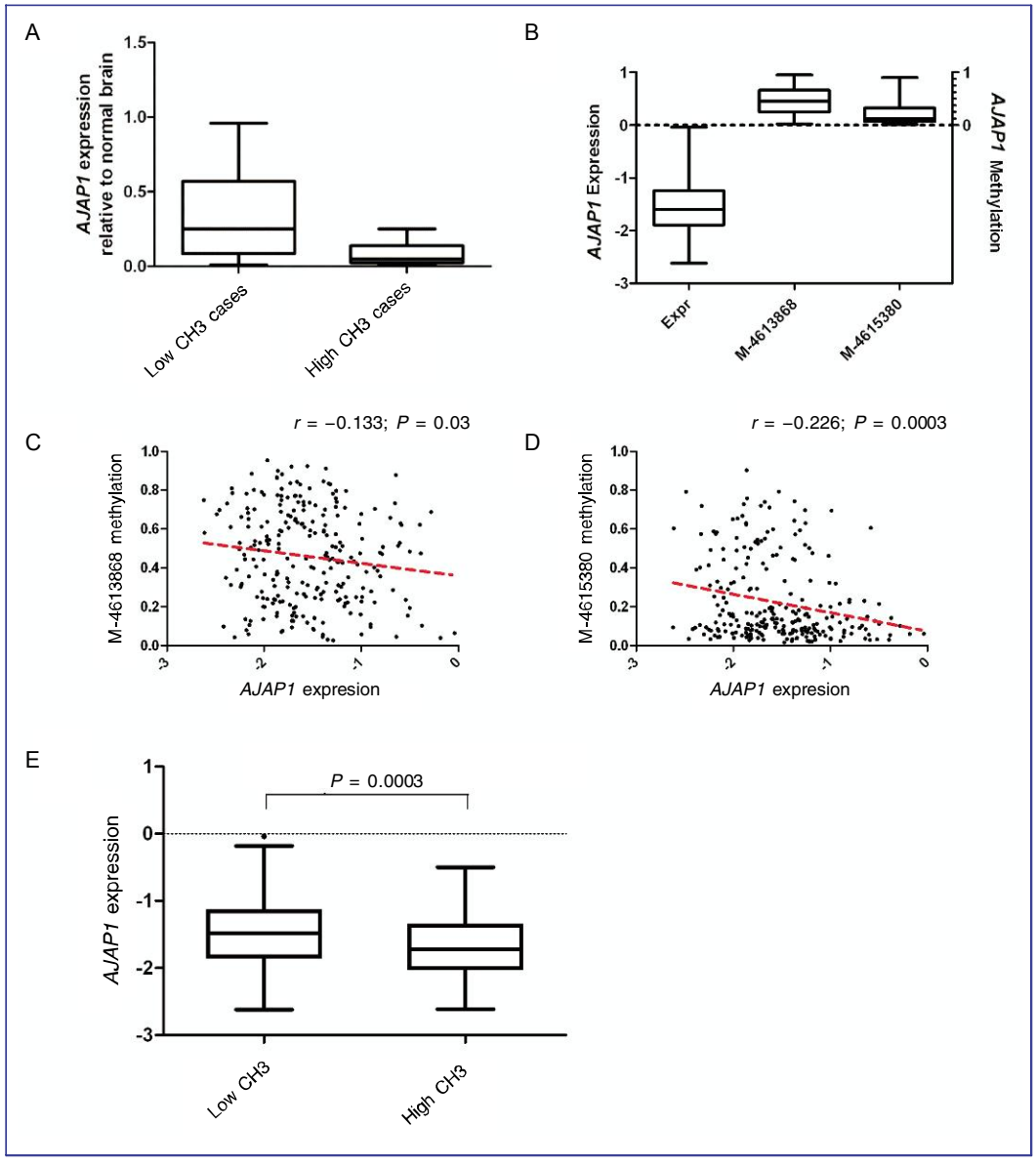


Figure 3. Correlation between methylation and *AJAP1* expression. A, *AJAP1* expression in 35 brain tumors was stratified into four groups according to the average methylation status of the cases. The 6 cases in the group with >15% methylation were considered to be in the high-methylation category and had the lowest level of expression. B, distribution of *AJAP1* expression and methylation across the 253 glioblastoma samples in the Cancer Genome Atlas database. C, correlation of *AJAP1* expression with methylation at M-4613868. D, correlation of *AJAP1* expression with methylation at M-4615380. The dashed red lines denote the linear regression of correlation; *r:* Pearson coefficient. E, *AJAP1* expression in the low-CH3 and high-CH3 groups based on the methylation values at M-4615380 using the same CH3 cutoff of 15%. CH3 stands for methylation.

expression and methylation is depicted in Figure 3B, which shows that *AJAP1* was down-regulated in the samples with a median value of about −1.60 (log2 ratio). Methylation was almost three times higher at M-4613868 (45.6%) than at M-4615380 (12.6%). Both M-4613868 ($r$ = −0.133, $P$ = 0.03) and M-4615380 ($r$ = −0.226, $P$ < 0.001) exhibited an inverse correlation with *AJAP1* expression (Figure 3C and 3D). In a manner similar to the one depicted in Figure 3A, we split the patients into two groups (144 with low methylation and 109 with high methylation) based on their values at M-4615380 using the same cutoff of 15%. Consistently, patients in the low CH3 group (median −1.479) exhibited higher *AJAP1* expression than those in the high CH3 group (median −1.718) with statistical significance ($P$ < 0.001) (Figure 3E). Methylation at M-4613868 was comparatively high, and it was difficult to split the patients into two distinct groups based on the same CH3 cutoff. In all, we experimentally and computationally observed a statistically significant correlation between methylation of the *AJAP1* promoter and *AJAP1* expression. Using an independent dataset of 343 glioma samples profiled in the REMBRANDT application (http://rembrandt.nci.nih.gov)[26], we determined the association between *AJAP1* expression and survival (Figure 4). This relationship indicates that the *AJAP1* gene product affects glioma progression[18].

Thus, we propose that *AJAP1* is a putative tumor suppressor gene located on 1p36 and inhibits cell adhesion and migration. Although no tumor-specific mutations were observed, *AJAP1* may still fit the two-hit hypothesis for tumor suppressor genes in that expression from two wild-type alleles is abrogated by either gene deletion or promoter hypermethylation, or in the cases where gene deletion is absent, both hits can be manifested by promoter hypermethylation. Further investigations are needed to determine the role of *AJAP1* in the etiology of oligodendroglioma and other tumor types with nonrandom 1p36 allelic loss.

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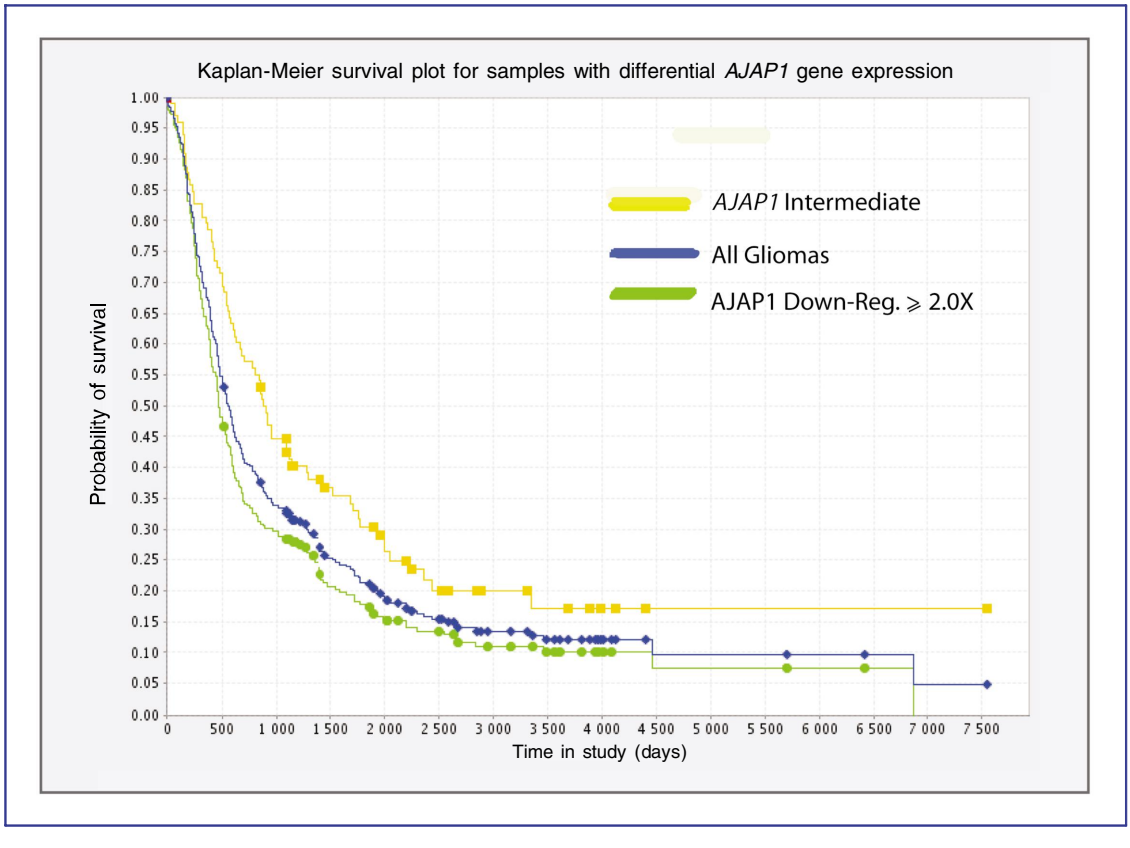


Figure 4. Association of *AJAP1* expression with survival using glioma data from REMBRANDT. Of the 343 patients with glioma, 245 had down-regulated *AJAP1* expression and 98 had up-regulated expression. The survival rate, calculated with the Kaplan-Meier method, was significantly lower in the patients with down-regulated *AJAP1* expression than in those with intermediate expression ($P$ = 0.001).

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