Down-regulation of RNA Editing in Pediatric Astrocytomas

ADAR2 EDITING ACTIVITY INHIBITS CELL MIGRATION AND PROLIFERATION

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Caterina Cenci1, Rita Barzotti1, Federica Galeano2, Sandro Corbelli3, Rossella Rota4, Luca Massimi5, Concezio Di Rocco6, Mary A. O’Connell6, and Angela Gallo4

From the RNA editing Laboratory, and Angiogenesis Laboratory, Ospedale Pediatrico Bambino Gesù Research Institute, Piazza S. Onofrio 4, 00165 Rome, Italy, the Pediatric Neurosurgery Policlinico Gemelli, Largo Agostino Gemelli, 8, 00168, Rome Italy, and the MRC Human Genetics Unit, Western General Hospital, Crewe Road, Edinburgh, EH4 2XU

Since alterations in post-transcriptional events can contribute to the appearance and/or progression of cancer, we investigated whether RNA editing, catalyzed by the ADAR (adenosine deaminases that act on RNA) enzymes, is altered in pediatric astrocytomas. We find a decrease in ADAR2 editing activity that seems to correlate with the grade of malignancy in children. Despite the loss of ADAR2 editing activity in tumor tissues, the high grade astrocytomas do not exhibit alterations in ADAR2 expression when compared with their specific control tissues. However, high expression levels of ADAR1 and ADAR3 were found in tumors when compared with normal tissues dissected in the same area of the brain. We reintroduced either ADAR2 or the inactive version of ADAR2 in three astrocytoma cell lines (U118, A172, U87). The “reverted” editing status is necessary and sufficient for a significant decrease in cell malignant behavior as measured by proliferation, cell cycle, and migration assays. We show that elevated levels of ADAR1, as found in astrocytomas, do indeed interfere with ADAR2 specific editing activity. Furthermore, we show that the endogenous ADAR1 can form heterodimers with ADAR2 in astrocytes.

The ADARs3 (adenosine deaminases that act on RNA) are enzymes responsible for adenosine (A) to inosine (I) conversion in pre-mRNA. This deamination is the most widespread type of RNA editing in higher eukaryotes. This deamination is the most widespread type of RNA editing in higher eukaryotes. The on-line version of this article (available at http://www.jbc.org) contains three supplemental figures.

The ADAR family includes three members in mammals. ADAR1 and ADAR2 are widely expressed and catalytically active (2–4), whereas ADAR3 is exclusively expressed in the brain and is inactive on all substrates tested (5, 6). There are two isoforms of ADAR1, a longer 150-kDa protein that is interferon-inducible and a 110-kDa protein that differs at the amino terminus and is constitutively expressed. The ADARs bind double-stranded RNA (dsRNA) formed between a sequence containing an editing site and a downstream or upstream editing complementary sequence (7). All ADARs have a similar domain structure with a catalytic domain at the carboxyl terminus and two or three dsRNA binding domains at the amino terminus. It has been reported that they can form dimers (8–11). The edited mammalian transcripts that have been best characterized are expressed in the central nervous system and include transcripts encoding the subunits GluR-B, GluR-C, and GluR-D of the α-amino-3-hydroxy-5-methyl-4-isoxozolepropionate receptor (AMPA), the subunits GluR-6 and GluR-5 of the kainate receptor (Kainate), and the serotonin receptor (5-HT2C) (for a review, see Refs. 12 and 13). The transcripts encoding GluR-B (AMPA subunit) and GluR-6 (Kainate subunit) are essential for the intracellular Ca2+-wave propagation and neurotransmitter release and are also involved in neuron/glial differentiation (14, 15). GluR-B dominates major AMPA receptor transmission properties via arginine 607, which is not coded by its gene but generated by RNA editing. This editing changes the glutamine to arginine in all the GluR-B transcripts (100% editing efficiency), making this editing event unique. The inability to edit this site can be dramatic as ADAR2−/− mice are prone to seizures and die soon after birth (16). As most of the known ADAR substrates are expressed in the central nervous system, defects in ADAR activity have been observed mainly in neurological diseases such as amyotrophic lateral sclerosis, Alzheimer disease, Huntington disease, schizophrenia, stroke, and epilepsy (for reviews, see Refs. 13 and 17).

Astrocytomas are the most common tumor type affecting the central nervous system. They originate from astrocytes, a type of glial cell, and are classified into increasing grades of malignancy with the most aggressive form being grade IV, the glioblastoma multiforme (or GBM), which is almost invariably fatal (18). A decrease in editing at the GluR-B Gln/Arg site in astrocytomas is necessary and sufficient for a significant decrease in cell malignant behavior as measured by proliferation, cell cycle, and migration assays. We show that elevated levels of ADAR1, as found in astrocytomas, do indeed interfere with ADAR2 specific editing activity. Furthermore, we show that the endogenous ADAR1 can form heterodimers with ADAR2 in astrocytes.
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EXPERIMENTAL PROCEDURES

Tumor and Control Tissues—We included 14 tissue samples from 10 children (age range at the time of diagnosis 9–15 years). None of the patients previously received radio- or chemotherapy. Tumor tissue samples were dissected from the core of the tumors. Malignancy grade (from grade I to IV) was defined according to the guidelines of the World Health Organization (WHO) (see Table 1). For this study, we used only de novo (or primary) GBM. Low grade tumors were cystic astrocytomas with mural node and expansive growth (20). Mixed lineage, giant cell astrocytomas and gliosarcomas were excluded to avoid the presence of cell types other than astrocytes. To further decrease the possibility of contamination from cells other than astrocytes, we used only tissue samples with >90% astrocytes/10 random fields per slide. Control samples of normal white matter tissue were dissected close to the tumor during surgery. Astrocytes are particularly abundant in white matter. The study was approved by the local committee on the use of human samples for experimental studies.

RNA Isolation and Reverse Transcription—Total RNA was isolated with TRIzol reagent (Invitrogen) from the tumors, control brain tissues, cultured astrocytoma cell lines, and the HEK 293T cell line, according to the manufacturer’s instructions. The cDNA pools were generated by SuperScript II reverse transcriptase (Invitrogen) using random hexamer primers. The GluR-B cDNA encompassing both the Gln/Arg and the Arg/Glu editing positions was amplified by the following PCR primers: hGluR-Bf1, 5'-TTTAGCCTATGAGATCTGGATGTGC-3', hGluR-Brev, 5'-CAAGGTATGAAATCTCCAGCCAC-3'. For the analysis of the Ile/Val, Tyr/Cys, and Gln/Arg editing sites of the GluR-6, the following primers were used: hGluR-6fw, 5'-ACCTTGCAAGTGGCTCCACTGG-3'; hGluR-6rev, 5'-ATACGAAGAAATGATGATAAGTGG-3'.

Analysis of RNA Editing—Direct sequencing was performed on cDNA pools, and editing was calculated as described previously (21), or the PCR products were subcloned into the T-easy vector (Promega), and sequenced for each sample. A to G changes in the individual clones were analyzed. For each sample, 2–3 independent RT-PCR reactions were performed.

Astrocytoma Cell Line Transfection—Human astrocytoma cell lines A172 (ATCC CRL-1620™), U118 (ATCC HTB-15™), and U87 (ATCC HTB-14™) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Invitrogen) plus antibiotics. Stable polyclonal cell lines were generated by Geneticin selection using standard protocols. cDNA encoding the shorter and more active human isoform ADAR2a (henceforth ADAR2) was subcloned into pEGFP C3 vector (V-EGFP) (Clontech) with in-frame enhanced green fluorescent protein (EGFP) at the amino terminus. Geneticin-resistant cell clones (EGFP, EGFP-ADAR2, and EGFP-ADAR2 Glu/Ala) were generated and analyzed by Western blot using polyclonal anti-ADAR2 antibody (kindly provided by M. Ohman) or anti-EGFP (Clontech). All the cell lines generated in this study were stable polyclonal cell lines to avoid problems due to positional insertion effects. EGFP-ADAR2 and EGFP-ADAR2 Glu/Ala cell lines showed similar EGFP expression units (mean index fluorescent), and their RNA and protein levels were comparable as analyzed by real-time PCR and Western blot.

Cell Cycle Analysis by Flow Cytometry—1 × 10⁶ of A172, U118, and U87 cell lines stably transfected with EGFP, EGFP-ADAR2, and EGFP-ADAR2 Glu/Ala were trypsinized, washed with 1× phosphate-buffered saline, and fixed in 70% ice-cold ethanol. The cells were resuspended in phosphate-buffered saline 1× and then treated with RNase A (75 units/ml) for 30 min at 37 °C, and then propidium iodide was added at a 100 ng/ml final concentration. After staining, the cells were analyzed by a FACScalibur™ flow cytometer (BD Biosciences) using the CELLquest™ software (BD Biosciences) for both cell cycle profiles and apoptosis. Dead cells were excluded from analysis by side/forward scatter gating.

Proliferation Assay—5 × 10⁴ of A172, U118, and U87 cell lines and polyclonal cell lines stably transfected with EGFP, EGFP-ADAR2, and EGFP-ADAR2 Glu/Ala were seeded in 60-mm cell culture dishes. Alive adherent cells (Trypan blue dye exclusion) were determined daily, from day 1 to day 4. The assay was repeated four times in duplicates.

Cell Migration Assay—The Boyden chamber chemotaxis/migration assay was performed to measure cell migration. Poly-carbonate 8-μm filters were coated with 5 μg/ml gelatin, and NIH-3T3-conditioned medium was used as a chemo-attractant in the bottom chamber. The cell lines stably transfected with EGFP or with EGFP-ADAR2, and EGFP-ADAR2 Glu/Ala were placed in the upper compartment of the Boyden chamber at the density of 5 × 10⁴ or 1 × 10⁵ cells/well. After 4 h at 37 °C in 5% CO₂, the cells that had migrated to the lower surface were fixed, stained, and counted under a light microscope (×200 magnification). 10 random fields per slide were counted. Assays were carried out in triplicate and repeated at least four times in independent experiments.

Site-directed Mutagenesis to Generate Inactive EGFP-ADAR2 Glu/Ala—A single point mutation in the catalytic domain of ADAR2 was introduced using a site-directed mutagenesis kit (Stratagene) to change the amino acid Glu-396 into Ala-396 (changing the sequence from GAA to GCA). The oligonucleotides used for the mutagenesis were: hADAR2 Glu/Ala forward, 5'-GACTGCCATGCAGCAATAATACGCGG-3'; hADAR2 Glu/Ala reverse, 5'-CGAGATATTGCTGCATGCGCAGTC-3'. The PCR reaction was performed following the manufacturer’s instructions.

Editing Activity of ADAR2 and ADAR2 Glu/Ala in HEK 293T Cell Line—HEK 293T cells were chosen for the assessment of EGFP-ADAR2 and EGFP-ADAR2 Glu/Ala editing activity as the endogenous editing activity in this cell line is undetectable. They were transiently co-transfected independently with 4 and 8 μg of EGFP-ADAR2, EGFP-ADAR2 Glu/Ala, and pEGFP (V-EGFP) and 3 μg of GluR-B minigeneB13 (miniB13), which was used as the substrate. The miniB13 encodes a portion of the editing-competent murine glutamate receptor GluR-B gene that includes the Glu/Arg site that is edited by ADAR2. The PCR reaction was performed following the manufacturer’s instructions.
transfection was tested by both real-time PCR and Western blot.

**Sequence Analysis of ADAR1 and ADAR2 in Astrocytoma Cell Lines and Pediatric Astrocytomas**—For the sequence analysis of both ADAR1 and ADAR2 cDNAs, total RNA was isolated from cell lines and tissues as described, and the specific PCR products were analyzed by direct sequencing or subcloned into the T-easy vector (Promega), and 30–40 independent clones were sequenced. ADAR1 cDNA was reverse-transcribed using the hADAR1 8 reverse specific primer, hADAR1 8 reverse, 5′-CAGACAGATC-3′; ADAR2 cDNA was reverse-transcribed using the hADAR2 7 reverse, 5′-CCTGTGAGTTCA-GAATTTCTCAAGGGGCAGCTC-3′. For the PCR of the ADAR1, the following primers were used: hADAR1 4 forward, 5′-CCTGTAGTCTTCA-ACATGATAGAGC-3′; hADAR1 5 forward, 5′-GATAACT-TGGAAATCCATGATGCC-3′; hADAR1 8, reverse, 5′-CACA-TGGAGCATACATTGGAATCCATGATGCC-3′; ADAR2 cDNA was reverse-transcribed with hADAR2 7 reverse, 5′-ATGTCTGCCGGGT-CAGGGGGC-3′. For the ADAR2, the following primers were used: hADAR2 2 forward, 5′-GTATTTGCCATGGATATAGAAG-CTGGG-3′; hADAR2 4, forward 5′-GGAGAGCCATGCCAAGACTTTC-3′; hADAR2 6 reverse, 5′-TCAGGGGGCTGAG-TGAGAACCCTGTC-3′.

**Analysis of ADAR Expression**—Quantitative Real-time PCR was performed on all ADAR1, ADAR2, and ADAR3 mRNAs. Gene-specific exon-exon boundary PCR products (TaqMan gene expression assays, Applied Biosystems) were measured by means of a PE Applied Biosystems PRISM 7700 sequence detection system during 40 cycles. β-actin RNA or glyceraldehyde-3-phosphate dehydrogenase RNA were used for normalization, and relative quantification of gene expression was performed according to the 2^-ΔΔCt method (35). Real-time assays were repeated in triplicates from two independent RTs. The primers used were supplied by Applied Biosystems: ADAR1, all isoforms, identification number (ID) HS00241666_m1 spanning exons 2-3 boundary (NM_001105107.1); ADAR1 p150 ID HS01020780_m1, exons 1A-2 (NM_001111.3); ADAR2, ID HS00953730_m1, exons 5-6 (NM_001112.2); ADAR3, ID HS01024866_m1, exons 9-10 (NM_018702.1); β-actin, ID HS9999905_m1; glyceraldehyde-3-phosphate dehydrogenase, ID HS9999905_m1.

**Competition Experiments**—7 × 10^5 HEK 293T cells at 60% confluence were co-transfected with constant amounts of the GluR-B miniB13 (3 μg) and of EGFP-ADAR2 (8 μg) so that 50% editing of the Gln/Arg site was obtained, and an increasing amount of EGFP-ADAR1 (4, 8, 16, and 24 μg). pEGFP (V-EGFP) was also transfected so that the total concentration of exogenous DNA transfected was equal (total DNA transfected at each experimental point was 35 μg). After 24 h, the cells were collected, and cDNA was analyzed for RNA editing. The transfection was tested by real-time PCR and Western blot.

**Immunoprecipitation and Immunoblotting**—Total protein extracts from both wild type astrocytoma cell lines (U118 and A172) and those stably transfected were isolated with lysis buffer (150 mM NaCl, 25 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40, 0.1% SDS, 0.1 mM dithiothreitol) in the presence of a protease inhibitor mixture (Sigma). Total proteins were extracted from frozen tumor samples by homogenizing the tissues in lysis buffer (1% Nonidet P-40, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl, pH 7.8) in the presence of protease inhibitor mixture (Sigma). The immunoprecipitation assay was performed as described previously (22).

**Antibodies**—The antibodies used in this study were: anti-ADAR1 (monoclonal antibody 15.8.6, kind gift of K. Nishikura), anti ADAR2 (kind gift of M. Ohman), anti-EGFP (Clontech), and anti-tubulin α (Abcam). Anti-rabbit and antimouse horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology. The polyclonal antibodies raised against ADAR1 were produced in our laboratory in rabbit using a full-length 110-kDa ADAR1 protein glutathione S-transferase-tagged and overexpressed in *Escherichia coli*.

**Deletion Analysis of ADAR1 in Astrocytomas and Astrocytoma Cell Lines**—Deletion analysis of ADAR1 exon 2 was performed on total RNA treated with DNase and on genomic DNA (DNA Qiagen kit) from cell lines and tissues, and the primers used were: ADAR1 exon 2 reverse, 5′-AGTCCCAAGTTTCC-CAGACAGATC-3′; ADAR1 exon 2 forward 5′-AATA-GAATTCTCAAGGGGCAGCTC-3′.

**RESULTS**

**Decreased RNA Editing in Pediatric Astrocytomas**—RNA editing analysis was performed on both GluR-B and GluR-6 substrates in pediatric astrocytomas and controls. Although the GluR-B Gln/Arg site is always edited at 100%, the RNA editing of other sites varies with age and in different regions of the brain (23). Therefore, we tested patients with a limited age range (Table 1), and results were compared from each tumor tissue with the normal tissue dissected from the same area of the brain. Each tissue sample was identified with an ID (Table 1). Importantly, levels of editing among normal control tissues varied dependent upon which area of the brain they were dissected from (see differences in editing in control tissues in Table 1, highlighted in bold and underlining). We found 100% editing at the Gln/Arg site of the GluR-B transcript in controls and in low grade astrocytomas. Among the high grade tumors, four out of six showed a mild decrease in editing activity at this site (Table 1). The RNA editing of the GluR-B transcript at the Arg/Gly site and of the GluR-6 transcript at the Ile/Val, Tyr/Cys, and Gln/Arg sites varied among different regions of the brain, as expected from observations in mice (24). The percentage of editing at all the sites analyzed was lower in astrocytomas when compared with the corresponding normal control tissue.

The relationship between grade of malignancy and percentage of editing at the GluR-B Arg/Gly site and at the GluR-6 Ile/Val, Tyr/Cys, Gln/Arg sites was evaluated with a regression analysis. For this analysis, RNA editing in pediatric tumors was expressed as a percentage of the editing in the corresponding normal control tissue. The decrease in RNA editing was correlated with the grade of malignancy of the tumors (R2 values ranging from 0.457 to 0.714 at the four editing sites), with the high grade tumors showing lower editing (Fig. 1).

**Extra Copies of ADAR2 Enzyme Restores RNA Editing in Astrocytoma Cell Lines**—Since GluR-6 and GluR-2 transcripts are mainly edited by ADAR2 (16, 25), the decrease in RNA editing at these sites suggests an alteration in ADAR2 activity in pediatric astrocytomas. To investigate whether the loss of ADAR2 editing activity played a role in astrocytomas, we
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TABLE 1
Summary of all the tumor and control tissues analyzed in this study identified by ID numbers

| Location                  | ID sample | WHO grades and controls | Age | GluR-B % | GluR-6 % | GluR-B 6% | GluR-6 % |
|---------------------------|-----------|-------------------------|-----|---------|---------|-----------|---------|
|                           |           |                         |     | Gln/Arg | Arg/Gly | Ile/Val   | Tyr/Cys  |
|                           |           |                         |     |         |         |           |         |
| Sopratentorial astrocytomas | 1        | C                       | 15  | 100     | 77      | 71.7      | 73.9    |
|                           | 2        | T                       | 15  | 100     | 56.6    | 42.5      | 50      |
|                           | 3        | C                       | 12  | 100     | 75      | 67.3      | 81.6    |
|                           | 4        | III                     | 12  | 92      | 29.6    | 36        | 44      |
|                           | 5        | IV                      | 10  | 97      | 3.8     | 2.2       | 4.5     |
|                           | 6        | C                       | 15  | 100     | 8       | 30.8      | 32      |
|                           | 7        | T                       | 15  | 100     | 8       | 7.2       | 14.5    |
|                           | 8        | IV                      | 10  | 98      | 3.8     | 2.2       | 4.3     |
| Frontal lobe              |           |                         |     |         |         |           |         |
| Subtentorial astrocytomas | 9        | C                       | 14  | 100     | 92.3    | 45.4      | 60.6    |
|                           | 10       | T                       | 14  | 100     | 67      | 18.8      | 47      |
|                           | 11       | IV                      | 12  | 100     | 42.4    | 8.3       | 25      |
|                           | 12       | I                       | 14  | 100     | nd      | 12.5      | 12.5    |
|                           | 13       | III                     | 9   | 100     | 15      | 9         | 1       |
|                           | 14       | IV                      | 9   | 94      | 22      | 1         | 1       |

FIGURE 1. Correlation of RNA editing and grade of malignancy. RNA editing at the GluR-B Arg/Gly site and at the GluR-6 Ile/Val, Tyr/Cys, Gln/Arg sites (samples from ID 1 to ID 11; Table 1) are expressed as a percentage of the editing in control tissue (y axis) in different regions of the brain and plotted against the degree of malignancy (x axis). As tissue controls, we used the mean editing level of ID 1 and ID 3 for the temporal lobe, ID 6 for the frontal lobe, and ID 9 for the cerebellum. Analysis was performed using linear regression; p values and R² coefficients are shown for each editing site.

reverted the RNA editing status in three astrocytoma cell lines (U118, A172, and U87) by ADAR2 overexpression. Astrocytoma cell lines showed an undetectable or very low level of RNA editing, similar to the supratentorial high grade tumors at all the editing sites in both GluR-B and GluR-6 transcripts (see below and data not shown), including the Gln/Arg site of the GluR-B (Fig. 2B). Polyclonal cell lines stably transfected with ADAR2 fused with EGFP at the amino terminus were generated in U118, A172, and U87. As a control, EGFP was also transfected, and stable polyclonal cell lines were generated. Here we show results for the U118 cell line (Fig. 2), but similar results were also obtained with A172 and U87 transfected cell lines (see supplemental Fig. 1). The transfected EGFP-ADAR2 was analyzed by real-time PCR and Western blot analysis in all the three cell lines. The exogenous ADAR2 did not alter the expression of the other ADARs (Fig. 2A). The EGFP-ADAR2 fused protein was also analyzed using fluorescence microscopy, and the correct nucleolar localization of the EGFP-ADAR2 (26) was observed (Fig. 2C, panels V and VI). EGFP-ADAR2 enzymatic activity was determined by direct sequencing on cDNA pools of the endogenous GluR-B and GluR-6 substrates. No random/nonspecific editing was found in these transcripts due to ADAR2 overexpression. However, the overexpression of the EGFP-ADAR2 restored the RNA editing at all the specific editing sites in both GluR-B and GluR-6. In particular, the EGFP-ADAR2 fully restored the editing level at the GluR-B Gln/Arg site (100%) (Fig. 2B) and increased the editing level at the GluR-B Arg/Gly site (to ~70%), at the GluR-6 Glu/Arg site (to ~75%), at the GluR-6 Tyr/Cys site (to ~90%), and at the GluR-6 Ile/Val site (to ~40%), as analyzed by direct sequencing on cDNA pools. The editing levels observed in cells transfected with EGFP-ADAR2 were similar to the editing observed in control tissues dissected from the temporal lobe (Table 1) and demonstrates that the overexpressed EGFP-ADAR2 is active on endogenous substrates.

ADAR2 Inhibits Proliferation and Migration of Astrocytoma Cell Lines—To determine whether the overexpression of ADAR2 modulates cell growth in astrocytomas, a proliferation assay was performed on untransfected, stably transfected EGFP and EGFP-ADAR2 polyclonal cell lines (U118, A172, and U87). The EGFP-ADAR2 cell line, but not the control cell lines (untransfected and EGFP only), displayed a strong inhibition in cell proliferation (Fig. 2D). This difference was evident 48 h after seeding, and it was even more dramatic after 4 days, with >50% decrease in cell number (Fig. 2D). Similar results were observed using the A172 and U87 astrocytoma cell lines (supplemental Fig. 1A). Next, we studied the cell cycle to elucidate the differences in growth rate between ADAR2-overexpressing cell lines and controls. Flow cytometry analysis in the presence of propidium iodide was performed, and we found that cells overexpressing ADAR2 showed a different cell cycle profile when compared...
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**A**

FIGURE 2. Overexpression of ADAR2 in astrocytoma cell line. A, immunoblot analysis of total protein extracts from U118 cell line and from the polyclonal U118 cell lines stably transfected with EGFP and EGFP-ADAR2. The endogenous ADAR2 and the overexpressed EGFP-ADAR2 are indicated. B, sequence chromatograms of the endogenous GluR-B transcript in cell lines U118 and U118 stably transfected with EGFP and EGFP-ADAR2. An arrow indicates the Gln/Arg editing position. C, cell morphology changes after transfection with EGFP-ADAR2 (panel IV) when compared with the control (panel I). In particular, in panel I, we showed the shape of control astrocytoma cell line U118 EGFP. In panel II, we showed the diffuse distribution of the EGFP protein, and in panel III, we showed the merge image. In panel IV, we showed the shape of U118-EGFP-ADAR2 cell line. In panel V, we showed EGFP-ADAR2 specifically localized within the nucleolus, and in panel VI, we showed the merge image. Cultured cells were directly analyzed with a Nikon Eclipse TE200 inverted microscope, at ×400 magnification. D, cell proliferation was analyzed by measuring live cells for 4 days after seeding. Error bars indicate standard deviations of three independent experiments.

with the controls in astrocytoma cell lines U118, A172 (Fig. 3A), and U87 (supplemental Fig. 1C). In particular, a larger cell population was observed at the S/G2-phase, when ADAR2 was overexpressed, and this correlated with a decrease in the cell population at the G1-phase (Fig. 3A).

A migration assay on U118, A172, and U87 was subsequently performed. A Boyden chamber for the migration assay was used, and the number of cells that migrated through the porous membrane was counted. Once more, the overexpression of ADAR2 in astrocytoma cell lines clearly affected the migratory activity of the cells when compared with the controls, with a reduction of ~50% in migrating cells in the U118 ADAR2 cell line and ~80% in the A172 ADAR2 cell line (Fig. 3B) and a reduction of ~60% in migrating cells in the U87 cell line (supplemental Fig. 1B).

Active ADAR2 Deaminase Domain Is Necessary for the Rescue of the Cell Tumor Phenotype—To demonstrate that the reverted phenotype in astrocytoma cells was due to the ADAR2 editing activity, we generated an inactive ADAR2 by introducing a point mutation in the deaminase domain (Glu to Ala) (Fig. 4A) (27). The editing activity of EGFP-ADAR2 and the inactive EGFP-ADAR2 Glu/Ala was evaluated on the Gln/Arg site in GluR-B miniB13 in the HEK 293T cell line. The HEK 293T cells were chosen as they have undetectable endogenous editing activity. We verified that EGFP-ADAR2 Glu/Ala was inactive when compared with the active EGFP-ADAR2 (Fig. 4B). We then stably transfected the U118, A172, and U87 astrocytoma cell lines with the inactive EGFP-ADAR2 Glu/Ala. The correct nucleolar localization of EGFP-ADAR2 Glu/Ala in the stably transfected polyclonal cell line was confirmed by fluorescence microscopy (Fig. 4C). Real-time PCR and Western blot were also performed, showing that the overexpression of ADAR2 Glu/Ala did not alter the expression levels of other endogenous ADARs (supplemental Fig. 2B). The cell lines, with comparable expression levels of ADAR2 and ADAR2 Glu/Ala (supplemental Fig. 2B), were then analyzed for cell proliferation, cell cycle, and migration assays. The ADAR2 Glu/Ala cell lines behaved similarly to the control cell lines (untransfected or EGFP), with a big difference in both proliferation and migration when ADAR2 Glu/Ala cell lines were compared with the ADAR2 cell lines (Fig. 4, D and E, and supplemental Fig. 1, A–C). We then showed in three different astrocytoma cell lines U118, A172, and U87 that ADAR2 editing activity is necessary and sufficient for a significant decrease in cell malignant behavior as measured by proliferation and migration assays.

Unbalanced Expression among ADARs in Astrocytomas and Astrocytoma Cell Lines—Since no mutations in ADAR2 were found after sequencing cDNA pools isolated from pediatric GBMs and astrocytoma cell lines, we investigated whether the decrease in ADAR2 activity, found in this type of tumor, could be due to a decrease in ADAR2 mRNA expression. No significant differences in ADAR2 mRNA levels were found in tumors when compared with their controls. However, the expression of both ADAR1 and ADAR3 mRNAs was significantly higher in tumors when compared with controls from the same brain area. In particular, the expression of ADAR1 was elevated in supratentorial astrocytomas (Fig. 5A and see supplemental Fig. 3, A–D), whereas ADAR3 displayed an elevated expression in the subtentorial astrocytomas (supplemental Fig. 3E). The relative amount of ADAR1 mRNA expression seemed to increase strikingly with the severity of the tumor, being higher in the most aggressive tumors (ADAR1 expression levels in grade III and grade IV astrocytomas in supplemental Fig. 3, A–D). Specific oligonucleotides for the different ADAR1 isoforms were used to show...
that the inducible ADAR1 150-transcript was the one being highly expressed in vivo in high grade astrocytomas and astrocytoma cell lines (data not shown). However, when Western blot analysis was performed to confirm the elevated level of ADAR1, we found ADAR1 110-kDa isoform being highly expressed in tumors (Fig. 5A) and cell lines (supplemental Fig. 2A). To resolve this apparent contradiction, we sequenced the amino terminus of the ADAR1 cDNAs from both tumors and cell lines. A 136-nucleotide deletion within the ADAR1 exon 2 was identified in between 30–70% of the clones analyzed for each tumor and astrocytoma cell line. A 136-nucleotide deletion within the ADAR1 exon 2 was identified in between 30–70% of the clones analyzed for each tumor and astrocytoma cell line. To test this hypothesis, competition experiments were performed at the HEK 293T cell line. Constant concentrations of GluR-B miniB13 and EGFP-ADAR2 were co-transfected to obtain ~50% of Ala to Ile conversion at the Gln/Arg site within the miniB13 transcript, and increasing amounts of EGFP-ADAR1 (from 4 to 24 μg) were added. The EGFP vector was transfected so as to obtain the same concentration of total DNA transfected for each experimental point (Fig. 6A). We found that at a high concentration, ADAR1 can indeed decrease the editing at the GluR-B Gln/Arg site specifically edited by ADAR2 (Fig. 6B). To determine whether a high level of ADAR1 can “sequester” ADAR2 by heterodimer formation in astrocytes, co-immunoprecipitation experiments were also performed. We found that the ADAR1 110-kDa protein, highly expressed in high grade tumors and cell lines (Figs. 2A and 5B), can indeed form heterodimers with endogenous or exogenous ADAR2 (Fig. 6C). This is independent of the active deaminase domain as the inactive ADAR2 can also dimerize with ADAR1 (Fig. 6C). Due to the low levels of ADAR1 150-kDa protein, we were unable to ascertain whether ADAR2 could also co-immunoprecipitate this protein.

**DISCUSSION**

Neoplasms of astrocytes represent the most common tumors of the central nervous system, with GBM being the most aggressive form in both adults and children. Pediatric high grade astrocytomas are extremely rare and show peculiar clinical and pathological features different from the adult high grade astrocytomas (28). To ascertain whether RNA editing was altered in pediatric brain tumors, we studied pediatric astrocytomas at different grades of malignancy.

We observed a decrease in the ADAR2-mediated RNA editing in all the sites analyzed in the GluR-B and GluR-6 transcripts (Table 1). This decrease seems to correlate with histological malignancy, with editing being lower in GBMs and astrocytomas III (Fig. 1).

It should be noted that analysis of the GluR-B transcript in pediatric astrocytomas III and GBMs revealed only a modest decrease of RNA editing at the GluR-B Gln/Arg site in children (92% to 100%) (Table 1), when compared with adult GBMs (70% to 95%) (data not shown). The editing at the GluR-B Gln/Arg site in GBMs that we found in adult patients is in agreement with a previous study (19). Differences in RNA editing at this site between adult and pediatric high grade astrocytomas could be important as full editing at this site rescues the neurological phenotype in the Adar2−/− mice (16). Previous work by Ishiuchi et al. (29) showed that the loss of editing at this site increased astrocytoma invasiveness in vivo. However, we found that, in contrast to adults, the GluR-B Gln/Arg site in children is almost unaltered in GBMs, highlighting the importance of the RNA editing at other sites in pediatric astrocytomas.

To investigate whether a reverted RNA editing level could revert the cell cancer behavior, ADAR2 editing enzyme was
overexpressed in three astrocytoma cell lines, increasing the editing at all the sites of both GluR-B and GluR-6. Interestingly, the reverted editing status correlated with a reverted malignant behavior in all the astrocytoma cell lines. The morphology of the stably ADAR2 transfected astrocytoma cell lines changed from a long fusiform shape to a polygonal and more flattened shape resembling a normal astrocyte cell (Fig. 2C, compare panel I with panel IV). A proliferation assay was performed demonstrating that overexpression of ADAR2 consistently decreased the cell growth rate (Fig. 2D). Cell cycle analysis showed that the overexpression of ADAR2 enzyme arrested the cell cycle at the S-G2 phase in both the U118 and the A172 cell lines (Fig. 3A) and the U87 cell line (supplemental Fig. 1C) with no alteration in apoptosis being observed (data not shown). These experiments suggested that ADAR2 affects cell growth through the modulation of the cell cycle and not through apoptosis. As GBMs are high proliferative tumors and typically invade distal portions of the brain, a migration assay was also performed. A marked reduction in cell migration was observed only in cell lines overexpressing ADAR2. The overexpression of ADAR2 produced ∼50% decrease in cell proliferation and ∼50–80% decrease in cell migration in all three astrocytoma cell lines (Fig. 4, D and E, and see supplemental Fig. 1A and B). Interestingly, the overexpression of p53 in human melanoma cells produced ∼50–80% decrease in cell proliferation and ∼50% decrease in cell migration (30).

To prove that the effects on tumor cell growth and migration were due to the RNA editing activity, an inactive ADAR2 stable cell line (EGFP-ADAR2 Glu/Ala) was generated in U118, A172,

![Figure 4](https://example.com/figure4.png)
and U87 that behaved similarly to the untransfected cell lines in both cell proliferation and cell migration assays (Fig. 4, D and E, and supplemental Fig. 1, A–C). Therefore, we showed that ADAR2 editing activity is crucial for cell proliferation and migration and that it modulates the cell cycle at the S/G2 to G1 transition.

No significant alteration of mRNA sequence or level was observed in ADAR2 in tumors, control tissues, or astrocytoma cell lines. In contrast, a marked increase in ADAR1 (in supratentorial tumors and cell lines, Fig. 5, A and B, and see supplemental Fig. 2A) and ADAR3 (in subtentorial tumors, supplemental Fig. 3E) mRNA expression was observed in tumors when compared with their controls. The high expression of ADAR1 competes with ADAR2 editing activity at the Gln/Arg site. C, ADAR1 co-immunoprecipitates (IP) ADAR2 that is either overexpressed or endogenously expressed. The reverse experiment with the overexpressed ADAR2 is also shown.

FIGURE 5. Expression analysis of ADARs. A, an example of the expression level of ADARs mRNA isolated from the pediatric supratentorial high grade astrocytoma and the control sample. The real-time PCRs were repeated twice from two independent RTs for each sample. Error bars indicate standard deviations of two independent experiments. The folds of expression were represented in arbitrary units calculated as a relative-fold increase in expression when compared with the control samples and arbitrarily set to 1. Tumor sample and control were normalized to β-actin mRNA. B, Western blot analysis on the pediatric supratentorial high grade astrocytoma and controls from the same brain area, with the white matter indicated with C (ID 1), and gliosis. The 110-kDa ADAR1 isoform overexpressed in these tumors is indicated. C, deletion analysis of ADAR1 exon 2 in astrocytomas. RT-PCR on ID 2 (grade II), ID 4 (grade III), and ID 5 (grade IV) is shown. A full-length PCR product from the full-length exon 2 is shown (373 nucleotides (nt)) as well as a smaller product of 237 nucleotides that carries a 136-nucleotide deletion. From the same samples, we performed PCR directly on the genomic DNA (non-diluted DNA, nd) and at different DNA dilutions (dil. 1:5, 1:10, 1:50, MW, molecular weight, D). diagram showing the deletion within exon 2 of ADAR1 mRNA and protein products. The deletion within the exon 2 produces a frameshift within the 150 ADAR1 transcript. The first possible downstream ATG produces the 110 ADAR1 isoform.

FIGURE 6. Substrate competition and co-immunoprecipitation experiments between ADAR1 and ADAR2. A, summary of the competition experiment in which the miniB13 transcript was edited to 50% by ADAR2 and an increased concentration of ADAR1 interfered with the ADAR2 mediated Gln/Arg site editing. V-EGFP (pEGFP vector); B, sequence chromatograms of the miniB13 transcript, with the Gln/Arg site indicated. High expression of ADAR1 competes with ADAR2 editing activity at the Gln/Arg site. C, ADAR1 co-immunoprecipitates (IP) ADAR2 that is either overexpressed or endogenously expressed. The reverse experiment with the overexpressed ADAR2 is also shown.
Recently, another study was published confirming a general decrease in ADAR1 editing not only in human brain tumors but also in testis, prostate, lung, and kidney tumors, using mainly a bioinformatics approach (31). In agreement with this study, we also found a decrease in editing that seems to correlate with the grade of malignancy in pediatric astrocytomas. Furthermore, both studies showed that the overexpression of ADAR2 in astrocytoma cell lines slows down the proliferation rate of the cells. Here we went further, demonstrating that ADAR2 activity decreases the cell proliferation through the modulation of the cell cycle and that an active ADAR2 is also responsible for a decrease in cell migration. However, conversely, we found that ADAR1 was highly expressed in high grade astrocytomas and in the three different astrocytoma cell lines analyzed (U118, A172, and U87) by real-time PCR (Fig. 5A and see supplemental Fig. 3) and Western blot analysis in both tissues and cell lines (Fig. 5B for the tumor tissues and see supplemental Fig. 2A for the astrocytoma cell lines). Differences in the two studies could be due to different tissue controls used for the real-time PCR. In this regard, we showed that correct normal controls were extremely important in in vivo studies as big differences in editing and ADAR expression levels were found in different areas of the brain (see the comparison of sopperntorial and subtentorial samples in supplemental Fig. 3 and in Table 1). Alternatively, the apparent contradiction could also be due to the age of the patients analyzed as differences exist between adult and pediatric astrocytomas.

It is worth noting that in high grade astrocytomas, the increase in ADAR1 transcript that we found does not lead to the production of the full-length 150-kDa protein but rather the 110-kDa protein. We demonstrated that this is due to a splicing event leading to a deletion within exon 2 of the ADAR1 transcript in tumors. This splicing event and the subsequent higher production of the 110-kDa protein is more marked in high grade tumors (Fig. 5, B and C). We hypothesized that a high level of ADAR1 could affect the specific editing activity of ADAR2. This hypothesis was also supported by the observation that astrocytomas with higher ADAR1 expression showed a lower ADAR2 editing activity in vivo (Fig. 5A and Table 1 and see supplemental Fig. 3). Therefore, we tested this hypothesis by substrate competition assay and by studying heterodimer ADAR1/ADAR2 formation in astrocytoma cell lines. We observed that high concentrations of ADAR1 interfered specifically with the editing at the GluR-B Gln/Arg site, demonstrating that a correct balance between these two ADAR enzymes is critical for specific editing activity (Fig. 6). In addition, using both overexpressed and endogenous proteins, we found an ADAR2/ADAR1 heterodimer in astrocytoma cell lines (Fig. 6C). This heterodimer in astrocytomas appeared to be independent of the ADAR activity as the inactive ADAR2 can still form dimers (Fig. 6C). In Drosophila melanogaster, the formation of ADAR heterodimers significantly affects the editing activity in vitro and in vivo (9). Our results suggest that expression of the mammalian ADAR genes has to be finely tuned as an unbalanced ADAR expression could have important consequences on the final editing. Our work opens new insights into the knowledge of RNA editing, ADAR function, and their role in tumor malignancy and in other diseases in which ADAR2 is found to be down-regulated for no apparent reason such as amyotrophic lateral sclerosis (32, 33) or stroke (34).

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