Related to Testes-Specific, Vespid, and Pathogenesis Protein-1 (RTVP-1) Is Overexpressed in Gliomas and Regulates the Growth, Survival, and Invasion of Glioma Cells

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Abstract

In this study, we examined the expression and functions of related to testes-specific, vespid, and pathogenesis protein 1 (RTVP-1) in glioma cells. RTVP-1 was expressed in high levels in glioblastomas, whereas its expression in low-grade astrocytomas and normal brains was very low. Transfection of glioma cells with small interfering RNAs targeting RTVP-1 decreased cell proliferation in all the cell lines examined and induced cell apoptosis in some of them. Overexpression of RTVP-1 increased astrocyte and glioma cell proliferation and the anchorage-independent growth of the cells. In addition, overexpression of RTVP-1 rendered glioma cells more resistant to the apoptotic effect of tumor necrosis factor–related apoptosis-inducing ligand and serum deprivation. To delineate the molecular mechanisms involved in the survival effects of RTVP-1, we examined the expression and phosphorylation of various apoptosis-related proteins. We found that overexpression of RTVP-1 decreased the phosphorylation of c-Jun-NH2-kinase and increased the expression of Bcl2 and that expression of RTVP-1 decreased the phosphorylation of c-Jun. We further found that overexpression of RTVP-1 was correlated with the malignancy of astrocytic tumors and that RTVP-1 is a potent therapeutic target in gliomas. (Cancer Res 2006; 66(8): 4139-48)

Introduction

Gliomas are the most frequent primary brain tumors, accounting for >50% of all brain tumors (1, 2). Glioblastomas, the most malignant form, are characterized by increased proliferation and invasion into the surrounding normal brain tissue (3). Current treatment options include surgery, radiation therapy, and chemotherapy. Unfortunately, the prognosis of patients with glioblastomas remains extremely poor, and the median survival of 12 months from the time of diagnosis has not significantly changed during the last years (4, 5). Limitations to therapy include the infiltrative nature and enhanced angiogenesis of these tumors (6, 7). In addition, glioma cells are resistant to current modalities of irradiation, chemotherapy, and immunotherapy (8). Therefore, innovative approaches are essential for the treatment of patients with gliomas, especially because the occurrence of these tumors is increasing.

Related to testes-specific, vespid, and pathogenesis protein 1 (RTVP-1) is a novel gene that was cloned from human glioblastoma cell lines by two groups and was identified as glioma pathogenesis–related protein (9) or RTVP-1 (10). RTVP-1 was reported to be expressed in high levels in gliomas and glioma cell lines, whereas no expression was observed in other cells or tumors of the central nervous system (9, 10). In addition, RTVP-1 has also been implicated as a marker of myelomonocytic differentiation in macrophages (11) and has been reported to act as a tumor-suppressor gene in prostate cancer (12, 13). RTVP-1 contains a transmembrane domain and a putative signal peptide and it has high homology with the human testis-specific protein, TPX1 (14), the venom allergen, antigen 5 (15), and with a mouse acidic epididymal glycoprotein–like molecule (16). In addition, RTVP-1 is structurally related to group 1 of the plant pathogenesis–related protein (PR-1) proteins, which play major roles in plant defense response to viral, bacterial, and fungal infection (17, 18). Because the TPX-1 and the PR-1 proteins are all secreted (14, 18), it has been suggested that RTVP-1 is a secreted protein as well.

The functions of RTVP-1 have been mainly studied in prostate cancer cells (12, 13). RTVP-1 has also been implicated as a secreted protein, and its role in glioma cell function has not been reported. In this study, we show that the expression of RTVP-1 is correlated with the malignancy of astrocytic tumors, and that RTVP-1 is involved in the regulation of the proliferation, survival, and invasion of glioma cells.

Note: T. Rosenzweig and A. Ziv-Av contributed equally to this work.

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Materials and Methods

Materials. An affinity-purified monoclonal anti-FLAG antibody, leupeptin, aprotinin, phenylmethylsulfonyl fluoride (PMSF), and sodium vanadate were obtained from Sigma Chemical Co. (St. Louis, MO) and a polyclonal anti–protein kinase C antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Human tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) was from Peprotech (Rocky Hill, NJ), and anti-p-AKT, Akt, phosphorylated c-Jun-NH2-kinase (pJNK), JNK, poly(ADP)ribose polymerase (PARP), active caspase-3, Bax, and Bcl2 antibodies were obtained from Cell Signaling Technology (Beverly, MA).

Tumors and primary cultures. Tumors were collected from patients operated at Henry Ford Hospital (Detroit, MI). Tumors were classified according to the WHO criteria into the various subtypes of low-grade astrocytomas, anaplastic astrocytomas, and glioblastomas using formalin-fixed paraffin-embedded H&E-stained tissue slices. Fresh tissues were frozen immediately following surgery in liquid nitrogen and stored at −70°C until processing.

Primary cultures were obtained from freshly resected tissues immediately following 1 hour of surgical removal. Samples were first washed in PBS and then minced in small pieces in DMEM with 10% FCS and were further triturated to obtain maximal cell dispersion. Cells were plated in 25 cm2 tissue culture flasks and were grown for 7 to 10 days. Cultures were used up to passage 7.

Sample collection and processing were done according to the regulations of the committee on research involving human subjects of the Organization Institutional Review Board.

Cell lines. The glioma cell lines A172, U87, LN-18, T98G, and LN-229 were obtained from American Type Culture Collection (Manassas, VA), and the U251 and LN2308 were obtained from Oliver Bogler (Department of Neurosurgery, Henry Ford Hospital). Cells were grown on tissue culture plates in a minimum essential medium supplemented with 10% FCS and antibiotics. Cells were grown in an astrocyte-specific medium provided by Cambrex. Cells were grown on astrocyte–specific medium provided by Cambrex (Walkersville, MD). Cells were grown in an astrocyte–specific medium provided by Cambrex (Walkersville, MD).

RNA extraction and reverse transcription-PCR. Total RNA was extracted from the tissue samples by RNeasy (Qiagen, Santa Clarita, CA) according to instructions of the manufacturer. One microgram of total RNA was transcribed into cDNA using the Reverse Transcriptase System according to instructions of the manufacturer (Qiagen). Reverse transcription reaction was carried out using 2 μg total RNA as described for the RT-PCR analysis. A primer optimization step was tested for each set of primers to determine the optimal primer concentrations. Once the optimal primer concentrations were determined, primers, 25 μL of 2× SYBR Green Master Mix (Invitrogen, Carlsbad, CA), and 30 to 100 ng cDNA samples were resuspended in a total volume of 50 μL PCR amplification solution. The following primers were used: RTVP-1 forward, TGCCAGTTTCTCATAATA-CA; RTVP-1 reverse, GGATTTGGTCATACATGTT and S12 forward, TGGTGGGTTGTAATAGGAC; S12 reverse, CAAGCACAAGAAATGGCTG.

Reactions were run on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The cycling conditions composed of 4-minute polymerase activation at 95°C and 40 cycles, 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute. Cycle threshold (Ct) values were obtained from the ABI 7000 software. S12 or β-actin levels were also determined for each RNA sample as controls. Fold change of relative mRNA expression was determined using the 2−△△Ct method (19).

Northern blot analysis. Northern blotting was done on 10 μg total RNA by using 1% formaldehyde–agarose gel and transfer onto nylon membranes (Schleicher & Schuell, Dassel, Germany). DNA probes for the RTVP-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized by PCR and 32P-labeled by random priming (Biological Industries, Beit Haemek, Israel). Hybridization was done at 42°C in 5× SSC-50% formamide-5× Denhardt’s solution-2% SDS-10% dextran sulfate-100 μg denatured sheared salmon sperm DNA/mL. GAPDH probe was used to standardize loading.

Preparation of adenovirus. Northern blots were obtained from BD Biosciences (Mountain View, CA) and were similarly hybridized with the RTVP-1 and GAPDH probes.

Construction of RTVP-1 plasmids. The full-length RTVP-1 was amplified by RT-PCR on template of RNA obtained from U87 cells using specific primers (5’-TAAATCTCTTACATGCCATGAGTCTCA-3’ and 5’-TAAATCTCTTACATGCCATGAGTCTCA-3’). RTVP-1 was similarly cloned into the MTH vector (21). The PCR product was found to be identical to the published sequence of RTVP-1 (GI 1527074). The PCR product was cut with EcoR1 and cloned into pCMV-tag 2B (Stratagene, La Jolla, CA), which was previously constructed to contain the MluI site between the XhoI and the Apal sites (20). RTVP-1 was similarly cloned into the MTH vector (21). The integrity of the inserts was verified by DNA sequencing.

Cell transfection. Cells were transfected either with the control vector or with the RTVP-1 expression vector by electroporation using the Nucleofector device, protocol number A29 (Amza Biosystems, Gaithersburg, MD). Transfection efficiency using nucleofection was between 70% and 90%. Stable clones were isolated using selection medium containing 750 μg/mL geneticin (Life Technologies, Gaithersburg, MD). All the results were confirmed on one pool and an additional individual clone.

Small interfering RNA transfection. Small interfering RNA (siRNA) duplexes were synthesized and purified by Dharmacon (Lafayette, CO). The siRNA sequence for targeting RTVP-1 mRNA was 5’-AAGACTCGGGTCT- GAACTCCA-3’ (siRNA1). In addition, we used a pool of four RTVP-1 siRNA duplexes that was also obtained from Dharmacon. A pool of Bcl2 duplexes was obtained from Dharmacon and a scrambled sequence was used as a negative control. Transfection of siRNAs was done using OligofectAMINE (Invirogen) according to the instructions of the manufacturer. Experiments were done 72 hours after transfection.

Adenovirus preparation and infection. The AdEasy system was kindly provided by Dr. Vogelstein (The Johns Hopkins University School of Medicine, Baltimore, MD; ref. 22). Adenoviruses expressing RTVP-1 were prepared as previously described (23). Briefly, RTVP-1 was first cloned into the pShuttle-CMV. The linearized plasmid was transformed into Escherichia coli BJ5183-D-1 competent cells (Strategene) carrying the pAdEasy-1 plasmid that encodes the adenovirus-5 backbone. Recombination was confirmed by restriction and PCR analyses. The linearized recombinant plasmid was transfected into HEK293 cells, viruses were collected after 7 to 10 days and were further amplified. Cells were incubated with 5 multiplicity of infection of the recombinant adenovirus vectors for 1 hour. The medium
was then replaced with fresh medium and the cells were used 24 to 48 hours post infection.

**Generation of anti-RTVP-1 antibody.** Anti-RTVP-1 antibody was generated by 21st Century Biochemicals (Marlboro, MA) by immunizing rabbits with synthetic peptides VRHINKFRESVKPTAC and CVQLKYPNLIVLLD corresponding to amino acids 38 to 53 and 255 to 266 of human RTVP-1, respectively. The anti-RTVP-1 serum was affinity purified and characterized by ELISA and Western blot analysis.

**Quantitation of cell growth.** Cells (2 x 10^4) were seeded in triplicate in tissue culture dishes and were harvested at 24-hour intervals using trypsin. Cells were washed in PBS and were then counted using a phase microscope. Cell numbers were then calculated per milliliter of the original dilution. Each assay was done in quadruplicates.

**Soft agar assays.** Cells were harvested, washed, and plated in a soft agar (0.4%) overlaying a 0.9% base agar. The agar plates were incubated at 37°C for 10 days and colonies (containing >50 cells) were viewed by the microscope and counted. Each assay was done in triplicate.

**Preparation of cell homogenates and immunoblot analysis.** Cell pellets (10^6 cells/mL) were resuspended in 100 μL lysis buffer [25 mmol/L Tris-HCl (pH 7.4), 50 mmol/L NaCl, 0.5% Na deoxycholate, 2% NP-40, 0.2% SDS, 1 mmol/L PMSF, 50 μg/mL aprotinin, 50 μL/mL leupeptin, and 0.5 mmol/L Na_2VO_3] on ice for 15 minutes. Sample buffer (2×) was added and the samples were boiled for 5 minutes.

Lysates (30 μg protein) were resolved by SDS-PAGE and were transferred to nitrocellulose membranes. The membranes were blocked with 5% dry milk in PBS and subsequently stained with the primary antibody. Specific reactive bands were detected using a goat anti-rabbit or goat anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA) and the immunoreactive bands were visualized by the ECL Western blotting detection kit (Amersham, Arlington Heights, IL).

**Measurement of cell apoptosis.** Cell apoptosis was measured by flow cytometry after propidium iodide staining as previously described (23). Briefly, cells were treated according to the specific experiment. The cells were scraped and centrifuged with the supernatant medium at 3,500 rpm for 5 minutes. Cells were resuspended in PBS and fixed in 70% ethanol for 1 hour on ice. Fixed cells were washed with PBS and stained with propidium iodide (5 μg/mL) solution containing RNase (50 μg/mL). Cells were then analyzed on a Becton-Dickinson FACSCalibur.

Cell apoptosis was also measured using anhisthene ELISA (Cell Death Detection kit). For these experiments, extracts of cells containing histone-associated DNA fragments were incubated in 96-well plates coated with antihistone antibodies for 2 hours. Plates were then washed and incubated with anti-DNA antibodies conjugated to peroxidase for an additional 2 hours. Substrate solution was added and absorbance was measured at a wavelength of 410 nm. In addition, cell apoptosis was evaluated by Western blot analysis of PARP and caspase-3 cleavage using anti-PARP antibody and anti–active caspase-3 antibody.

**Invasion assay.** Boyden chamber chemoinvasion assays were done as previously described by Reich et al. (24). Matrigel (reconstituted basement membrane; 25 μg) was dried on a polycarbonate filter (polivinyl pyrrolidone-free; Nucleopore; Whatmann, Madistone, United Kingdom). Cells were harvested by brief exposure to 1 mmol/L EDTA, washed with DMEM containing 0.1% bovine serum albumin, and added to the Boyden chamber (200,000 cells). Cells were incubated for 6 hours at 37°C in humidified atmosphere of 95% air and 5% CO_2. Cells that transversed the Matrigel layer and attached to the filter were stained with Diff Quik kit (Dade Diagnostics, Aguada, Puerto Rico) and counted in five randomized fields. Results are expressed as the mean ± SE of three independent experiments.

**Analysis of matrix metalloproteinase isoforms (zymography).** Cells (200,000) were incubated for 24 hours in serum-free DMEM, and their supernatant was analyzed for matrix metalloproteinase (MMP) activity on a gelatin impregnated (1 mg/mL; Difco, Detroit, MI) SDS-PAGE 8% gel, as described previously by Reich et al. (24, 25). Culture medium samples were separated on substrate-impregnated gels under nonreducing conditions, followed by 30 minutes of incubation in 2.5% Triton X-100 (BDH, Poole, United Kingdom). Gels were then incubated for 16 hours at 37°C in 50 mmol/L Tris, 0.2 mol/L NaCl, 5 mmol/L CaCl_2, and 0.02% (w/v) Brij 35 (pH 7.6). At the end of the incubation period, gels were stained with 0.5% Coomassie G250 (Bio-Rad) in methanol/acetic acid/H_2O (30:10:60).

**Spheroid confrontation model.** The spheroid confrontation assay was done as previously described (26). Briefly, fetal rat brains were collected from Sprague-Dawley rats at embryonic day 18. Single-cell suspensions were made by digestion of whole brains in 0.1% tyrpsin/HBSS. Cell suspensions were cultured on agar-coated plates for 3 weeks to allow for the development of brain aggregates. Cells of interest are also cultured on agar coated plates for 2 weeks to allow for the development of spheroids. At the beginning of culture and once per week thereafter, either Cell Tracker Red or Cell Tracker Green (Invitrogen) was added to separate wells of aggregates and spheroids to allow for the visualization of confrontations. One aggregate and one spheroid were then transferred to a single agar-coated well of a 48-well culture plate and confronted mechanically. Confrontations were visualized at 24, 48, and 72 hours using an Olympus fluorescent microscope. Green and red fluorescence was visualized separately and merged to visualize invasion.

**Statistical analysis.** The results are presented as the mean values ± SE. Data were analyzed using ANOVA and a Student’s t test. For real-time PCR data, Student’s t test (with correction for data sets with unequal variances) was done using Prism 4 (GraphPad Software, Inc, San Diego, CA).

**Results**

**Expression of RTVP-1 in various human tissues.** We first examined the expression of RTVP-1 in various human tissues by RT-PCR and Northern blot analysis. Using RT-PCR, we found that the expression of RTVP-1 was very low in normal brain and undetectable in fetal brain. Similarly, RTVP-1 expression was not detected in the colon, pancreas, skin, lymphocytes, and fetal liver, whereas higher levels were detected in the heart, spleen, muscle, lung, bone marrow, placenta, adrenal gland, and prostate (Fig. 1A).

Using Northern blot analysis, we observed three main transcripts that were detected by the RTVP-1 DNA probe in the heart, placenta, liver, and skeletal muscle (Fig. 1B). This observation is similar to the results reported by Rich et al. (10). In line with the RT-PCR results, the expression of RTVP-1 in the brain was barely detected by the Northern blot analysis. In contrast, the expression of RTVP-1 in the lung was detected by RT-PCR but only weakly by Northern blot analysis. Similarly, five additional samples of lung tissues expressed RTVP-1 mRNA as detected by RT-PCR (data not shown), suggesting that the discrepancy in the results of the RT-PCR and the Northern blot analysis may represent differences in assay sensitivity or variability in RTVP-1 expression in different individuals.

**Expression of RTVP-1 in astrocytic tumors with different degrees of malignancy.** RTVP-1 has been previously reported to be expressed in glioblastomas (9, 10); however, its expression in other types of astrocytic tumors has not been reported. To characterize the expression of RTVP-1 in different types of astrocytic tumors, we used tumor samples from low-grade astrocytomas (grade 2), anaplastic astrocytomas (grade 3), and glioblastomas (grade 4). The expression of RTVP-1 in these tumors was compared with that of normal brains using semiquantitative RT-PCR. As presented in Fig. 2A, the expression of RTVP-1 in normal brains was barely detected. Similarly, most low-grade astrocytomas expressed very low levels of RTVP-1. In contrast, higher levels of RTVP-1 were observed in anaplastic astrocytomas and glioblastomas (Fig. 2A).

Quantitation of RTVP-1 expression compared with that of S12 (Fig. 2B). This observation is similar to the results reported by Rich et al. (10). In line with the RT-PCR results, the expression of RTVP-1 in the brain was barely detected by the Northern blot analysis. In contrast, the expression of RTVP-1 in the lung was detected by RT-PCR but only weakly by Northern blot analysis.

Similar results were obtained using real-time RT-PCR (Fig. 2C). Thus, the relative RTVP-1 mRNA expression to S12 in brains and
hybridization, membranes were rehybridized using a GAPDH probe to control for mRNA was also determined using Northern blot hybridization with RTVP-1.

Silencing of RTVP-1 decreases cell proliferation and induces cell apoptosis. To examine the role of RTVP-1 in glioma cell function, we first used siRNAs directed against the RTVP-1 mRNA. Transfection of the glioma cell lines, U87 and A172, with a siRNA duplex for 3 days resulted in around 90% and 70% decrease in the expression of the RTVP-1 mRNA, respectively, whereas no decrease in the expression of RTVP-1 was observed with a scrambled control siRNA (Fig. 3A). The RTVP-1 siRNA also decreased the expression of the RTVP-1 protein. Transfection of U87 and A172 cells with RTVP-1 siRNA significantly decreased the expression of RTVP-1 after 72 hours as detected by anti-RTVP-1 antibody, whereas a control siRNA did not have a significant effect (Fig. 3B).

The RTVP-1 siRNA–transfected U87 cells exhibited a significant decrease in cell proliferation (Fig. 3C) and in cell growth in soft agar (Fig. 3D). In addition, ~30% of the U87 cells were apoptotic as determined by propidium iodide staining and fluorescence-activated cell sorting (FACS) analysis (Fig. 3E). The A172 cells, in which RTVP-1 expression was less reduced by the siRNAs, exhibited a significant decrease in cell proliferation (Fig. 3C) and cell growth in soft agar (Fig. 3D), but a smaller degree of cell apoptosis as determined by propidium iodide staining (Fig. 3E). Similar results were observed with another set of RTVP-1 duplex (data not shown).

To further examine the effect of RTVP-1 silencing, we used established primary glioma cultures. Transfection of the primary cultures with RTVP-1 siRNAs reduced the expression of RTVP-1 mRNA (Fig. 3A) or protein levels (Fig. 3B) to a different degree. Similar to the results obtained with the glioma cell lines, cells in which RTVP-1 was significantly depleted (HF1308) exhibited both a decrease in cell number (Fig. 3C) and an increase in cell apoptosis (Fig. 3E). In contrast, cells in which RTVP-1 expression was only partially reduced (HF1254) showed a similar decrease in cell number but only a small increase in cell apoptosis (Fig. 3C and E).

Similar results of cell apoptosis were also observed using additional apoptosis assays, including anti–active caspase-3 antibody (Fig. 3F) and antihistone ELISA (Fig. 3G).

Overexpression of RTVP-1 increases glioma cell growth. Recent studies reported that overexpression of RTVP-1 in prostate cancer cell lines induced cell apoptosis in these cells (12, 13). To examine the effect of RTVP-1 on glioma cells, we used glioma cells stably expressing RTVP-1 (Fig. 4A). For these experiments, we used two clones of control vector cells, CV1 and CV2, and the RTVP-1-overexpressing cells, RTVP1-1 and RTVP1-2. Overexpression of RTVP-1 is presented in Fig. 4A using anti-FLAG antibody. We found that U87 cells stably expressing RTVP-1 (RTVP1-1 and RTVP1-2 cells) exhibited higher degree of cell proliferation compared with control vector (CV1 and CV2) cells (Fig. 4B). In addition, we found that overexpression of RTVP-1 enhanced the colony-forming ability of the U87 cells on soft agar (Fig. 4C and D). Thus, RTVP-1-overexpressing cells exhibited an increase in the size and number of colonies formed on the soft agar compared with the control vector cells, suggesting that RTVP-1 increased also the anchorage-independent growth of glioma cells. Similar results were obtained with two clones of A172 cells overexpressing RTVP-1 (data not shown).

Because RTVP-1 expression is higher in glioma cells compared with astrocytes, we examined the effect of RTVP-1 overexpression on the growth of glioma cells, which was examined using an anti-RTVP-1 antibody and Western blot analysis. The anti-RTVP-1 antibody recognized a band around 28 kDa and this band was blocked by the addition of the immunizing peptides. Similar to the mRNA results, RTVP-1 was highly expressed in glioblastoma, whereas low expression was observed in normal brains and low-grade astrocytomas (Fig. 2F). Thus, the relative RTVP-1 protein expression compared with actin was 4.8 ± 0.7 in glioblastomas, 0.56 ± 0.07 in low-grade astrocytomas, and 0.11 ± 0.2 in normal brains.

The expression profile of RTVP-1 in human tissues. The expression profile of RTVP-1 was determined in various human tissues by semiquantitative RT-PCR using the Human Rapid-Scan panel (Origene). The expression of S12 was used as a control for equal loading (A). The expression of RTVP-1 mRNA was also determined using Northern blot hybridization with RTVP-1 probe of the Multiple Human Tissue Northern Blots (BD Bioscience). After hybridization, membranes were rehybridized using a GAPDH probe to control for variations in gel loading and transfer efficiency (B). Representative of two similar experiments.

The expression of RTVP-1 was also examined in glioma cell lines and primary glioma cultures. As presented in Fig. 2D, RTVP-1 was expressed in all the glioma cells examined, whereas it was barely detected in human fetal astrocytes.

Using Northern blot analysis, we observed a strong expression of the 4.2, 3.2, and 1.1 kb transcripts in the U87 and A172 glioma cell lines and a weaker expression of the 1.6 kb transcript in these cells (Fig. 2E).

Previous studies reported the expression of four distinct mRNAs of 4.2, 3.2, 1.6, and 1.0 kb by using an RTVP-1 DNA probe. To further explore this point, we examined the effect of the RTVP-1 siRNA on the expression of the different mRNAs. We found that the RTVP-1 siRNA, which targets the first exon of RTVP-1, significantly reduced the expression of the 1.6 and 1.0 kb mRNAs, whereas it did not significantly affect the expression of the 4.2 and 3.2 transcripts (data not shown).

The expression of the RTVP-1 protein was examined using an anti-RTVP-1 antibody and Western blot analysis. The anti-RTVP-1 antibody recognized a band around 28 kDa and this band was blocked by the addition of the immunizing peptides. Similar to the low-grade astrocytomas was very low (1.30 ± 0.22 and 2.2 ± 0.34, respectively), whereas glioblastomas expressed significantly higher levels of RTVP-1 (6.9 ± 0.92).

Similar results of cell apoptosis were also observed using additional apoptosis assays, including anti–active caspase-3 antibody (Fig. 3F) and antihistone ELISA (Fig. 3G).

Overexpression of RTVP-1 increases glioma cell growth. Recent studies reported that overexpression of RTVP-1 in prostate cancer cell lines induced cell apoptosis in these cells (12, 13). To examine the effect of RTVP-1 on glioma cells, we used glioma cells stably expressing RTVP-1 (Fig. 4A). For these experiments, we used two clones of control vector cells, CV1 and CV2, and the RTVP-1-overexpressing cells, RTVP1-1 and RTVP1-2. Overexpression of RTVP-1 is presented in Fig. 4A using anti-FLAG antibody. We found that U87 cells stably expressing RTVP-1 (RTVP1-1 and RTVP1-2 cells) exhibited higher degree of cell proliferation compared with control vector (CV1 and CV2) cells (Fig. 4B). In addition, we found that overexpression of RTVP-1 enhanced the colony-forming ability of the U87 cells on soft agar (Fig. 4C and D). Thus, RTVP-1-overexpressing cells exhibited an increase in the size and number of colonies formed on the soft agar compared with the control vector cells, suggesting that RTVP-1 increased also the anchorage-independent growth of glioma cells. Similar results were obtained with two clones of A172 cells overexpressing RTVP-1 (data not shown).

Because RTVP-1 expression is higher in glioma cells compared with astrocytes, we examined the effect of RTVP-1 overexpression...
on cultured normal human astrocytes. For these experiments, we infected human astrocytes with an adenovirus vector expressing RTVP-1 and examined the proliferation of the cells following 1 to 4 days in culture. As presented in Fig. 4E, astrocytes expressing RTVP-1 exhibited higher degree of cell proliferation after 3 and 4 days in culture compared with astrocytes infected with an adenovirus vector expressing LacZ (control vector).

RTVP-1 regulates glioma cell survival. Because silencing of RTVP-1 induced cell apoptosis in the glioma cell lines, we examined the role of RTVP-1 overexpression in glioma cell survival using the apoptotic stimuli TRAIL and serum starvation. U87 cells overexpressing control vector cells (CV1 and CV2) exhibited ~30% of apoptotic cells in response to TRAIL (100 ng/mL). In contrast, the RTVP1-1 and RTVP1-2 overexpressors exhibited a significantly lower degree of cell apoptosis (Fig. 5A). In addition, we found that cells overexpressing RTVP-1 were more resistant to cell apoptosis induced by serum deprivation compared with control vector cells (Fig. 5B). Similar results were obtained using anti-PARP antibody, which detected PARP cleavage (Fig. 5C).

Similarly, RTVP-1 protected A172 from TRAIL-induced apoptosis. Thus, TRAIL induced apoptosis (24%) in the A172 cells after 5 hours of treatment, whereas A172 cells infected with adenovirus

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**Figure 2.** Expression of RTVP-1 in astrocytic tumors and glioma cell lines. Total RNA was extracted from normal brains, low-grade astrocytomas (grade 2), anaplastic astrocytomas (grade 3), and glioblastomas (grade 4). The expression of RTVP-1 was determined using semiquantitative RT-PCR (A and B) or real-time PCR (C) as described in Materials and Methods. Results of densitometry of the semiquantitative RT-PCR (relative RTVP-1 mRNA to S12) are presented in the graph for normal brains (n = 8), low-grade astrocytoma (LGA, n = 16), anaplastic astrocytomas (AA, n = 23), and glioblastomas (GBM, n = 33; B). *, P < 0.05; **, P < 0.001. Real-time PCR was done using RTVP-1 and S12 probes of mRNA from five normal brain tissues, 12 low-grade astrocytomas, and 15 glioblastomas. Results are normalized relative to the levels of S12 mRNA and are presented relative to reference sample. Mean values are marked (C). RNA was extracted from glioma cell lines, primary glioma cultures, and normal human astrocytes. RTVP-1 levels were determined using semiquantitative RT-PCR (D). The expression of RTVP-1 in the U87 and A172 cells was also determined using Northern blot analysis (E) as described in Fig. 1. After hybridization, membranes were rehybridized using a GAPDH probe to control for variations in gel loading and transfer efficiency. RTVP-1 protein levels were evaluated in seven glioblastomas, five low-grade astrocytomas, and two brains using Western blot analysis and anti-RTVP-1 antibody (F, 1:500 dilution). One representative of four similar experiments.
vector expressing RTVP-1 showed a significant lower level of cell apoptosis (9%; Fig. 5D).

To delineate the mechanisms involved in the survival effect of RTVP-1, we examined the expression and phosphorylation of the apoptosis-related proteins Bcl2, Bax, AKT, and JNK. We found that U87 cells overexpressing RTVP-1 (RTVP1-1 and RTVP1-2) expressed higher levels of Bcl2, whereas no significant differences were obtained in the expression of Bax. In addition, cells overexpressing RTVP-1 exhibited lower levels of pJNK, whereas no significant changes were observed in the phosphorylation or the expression of AKT between the two groups of cells (Fig. 5E).

To examine the role of Bcl2 in the protective effect of RTVP-1, we transfected the RTVP-1-overexpressing U87 cells (RTVP1-1) with Bcl2 siRNA and examined the response of the cells to the apoptotic effect of TRAIL. The expression of Bcl2 was significantly decreased in RTVP1-1 cells (Fig. 5F) and the CV1 cells (data not shown) transfected with the Bcl2 siRNA for 2 days. We found that silencing of Bcl2 slightly increased the response of the CV1 cells to the apoptosis induced by TRAIL, whereas it significantly increased the apoptosis of the RTVP-1-overexpressing cells from 12.4% to 22.5%. Thus, the increase in Bcl2 observed in the RTVP-1-overexpressing cells plays a role in the protective effect of RTVP-1 (Fig. 5G).

RTVP-1 regulates the invasion and MMP2 activity in glioma cells. Glioma cells are characterized by increased invasion and migration (27, 28). To examine the role of RTVP-1 in glioma cell invasion, we examined the ability of the cells to migrate through a Matrigel layer using Boyden chamber. Overexpression of RTVP-1 significantly increased the invasion of U87 cells by 3-fold compared with control vector cells (Fig. 6A). Similar results were observed in A172 cells overexpressing RTVP-1 and these cells exhibited a 4-fold increase in cell invasion compared with control vector cells (data not shown).

To further examine the effect of RTVP-1 on glioma cell invasion, we used the spheroid confrontation assay that reflects more the in vivo conditions (25). In this assay, astrocytic and glioma spheroids were coplated and the degree of invasion of the glioma

Figure 3. Silencing of RTVP-1 decreases cell growth and induces cell apoptosis. U87, A172, HF1254, and HF1308 cells were transfected with siRNAs targeting the RTVP-1 mRNA. The expression of RTVP-1 mRNA was determined after 3 days using semiquantitative RT-PCR (A). S12 was used to control for equivalent loading/amplication. The effect of the RTVP-1 siRNA on the RTVP-1 protein levels was examined in U87, A172, HF1308, and HF1254 cells using anti-RTVP-1 antibody and Western blot analysis after 3 days of transfection (B). For determining cell proliferation, cells were counted after 3 days of siRNA transfection (C). For the anchorage-independent growth of the cells, A172 and U87 cells transfected with control siRNA (Con-siRNA) and with RTVP-1 siRNA (RT1-siRNA) were plated in soft agar for 8 days and colonies were viewed under a phase contrast microscope and counted (D). The apoptosis of the cells was determined using propidium iodide staining and FACS analysis (E), by anti-active caspase-3 antibody (F) and by antihistone ELISA (G). Results are means of three independent experiments (C, D, E, and G) or one of four separate experiments that gave similar results (A, B, and F); bars, SE.
cells into the astrocytic spheroids was then determined. As presented in Fig. 6B, U87 overexpressing RTVP-1 started to invade the astrocytic spheroids within 24 hours of their confrontation, whereas the control vector cells only established a minimal contact with the astrocytic spheroids at that time. After 48 hours, the majority of the astrocytic spheroids were completely invaded by the RTVP-1 overexpressors, whereas only a partial invasion of the astrocytic spheroids was observed by the control vector cells (Fig. 6B). Similar results were obtained in additional three experiments (data not shown). Thus, overexpression of RTVP-1 increases the invasive response of glioma cells.

MMPs play important roles in the digestion of extracellular matrix and in the invasion of tumor cells (28, 29). Using a zymography assay, we examined the activation of MMP2 in conditioned medium of control vector and RTVP-1-overexpressing cells. In accordance with our results of the invasion assays, we found that RTVP-1 increased the activity of MMP2 in the U87 cells (Fig. 6C). U87 did not express MMP9 and overexpression of RTVP-1 did not induce the expression or activation of this MMP (Fig. 6C).

**Discussion**

In this study, we examined the expression of RTVP-1 in various astrocytic tumors and the role of RTVP-1 in glioma cell function. RTVP-1 has been originally cloned from human glioma cell lines (9, 10); however, its expression in human tissues and astrocytic tumors has not been fully characterized and its functions in glioma cells have not yet been reported.

Using RT-PCR and Northern blot analysis, we found that RTVP-1 was expressed in various human tissues, such as prostate, heart, spleen, and bone marrow, whereas its expression in other tissues, such as brain, colon, pancreas, and skin was very low or undetectable. These findings further extend the results reported by Rich et al. (10) that RTVP-1 is expressed in a large number of tissues and support their results that the expression of RTVP-1 in normal brain was absent or barely detected. The role of RTVP-1 in the different normal tissues in which it is expressed is currently not understood. Based on its homology to members of the PR-1 proteins (9, 17), RTVP-1 may be involved in immune responses to various pathogens.

Although RTVP-1 has been reported to be highly expressed in gliomas, its relative expression in astrocytic tumors with different degree of malignancy was not examined. Using semiquantitative RT-PCR, we found that the expression of RTVP-1 was the highest in glioblastomas (grade 4), whereas most low-grade astrocytomas (grade 2) expressed very low levels of RTVP-1. Similar results were obtained using Western blot analysis and anti-RTVP-1 antibody. Thus, our results show that RTVP-1 is highly expressed in gliomas, similar to previous reports (9, 10), and indicate that the expression of RTVP-1 is correlated with the degree of malignancy of the astrocytic tumors.

In agreement with previous reports (9, 10), three predominant mRNAs of 4.2, 3.2, and 1.0 kb and a weaker band of 1.6 kb were
detected by using an RTVP-1 DNA probe. Suppression of RTVP-1 protein expression was evident by using siRNA, which targets the first exon of RTVP-1. This was coupled with a selective knockdown of the expression of the 1.6 and 1.0 kb mRNAs. Our results, along with database from Genbank, imply for two RTVP-1-encoding transcripts of 1.6 and 1.0 kb; the first contains a longer 3' untranslated region. These transcripts are likely to be functionally equivalent although they could be differentially regulated and transcribed. The longer transcripts of 3.2 and 4.2 kb are likely to have partial overlap with RTVP-1 sequences, excluding exon 1. Therefore, they remained stable in the presence of siRNA, which targets the first exon of RTVP-1. These transcripts probably represent different genes, such as the 3,359 bases HIV-1 rev binding protein 2 mRNA, which contains sequences that are complementary to RTVP-1 exons 5 to 6 and is being transcribed on the opposite strand.

To delineate the function of RTVP-1 in glioma cells, we first used siRNAs targeting the RTVP-1 mRNA. Silencing of RTVP-1 expression decreased cell proliferation and anchorage-independent growth in the cell lines examined. In addition, the silencing of RTVP-1 induced cell apoptosis in some of the cell lines. Thus, cell lines and primary cultures in which RTVP-1 expression was almost completely reduced exhibited a large degree of cell apoptosis, whereas a small degree of cell apoptosis was observed in cells in which RTVP-1 expression was moderately silenced. Our results point to an important role of RTVP-1 in the control of glioma cell growth and survival and suggest that a decrease in the expression of RTVP-1 affects the proliferation of glioma cells and that even low levels of RTVP-1 are sufficient to maintain the survival of these cells. Alternatively, the differential apoptotic response of the glioma cells to RTVP-1 silencing may be due to differences in the expression of other apoptosis-related proteins or signaling pathways, such as p53 or Bcl2.

Our conclusions regarding the roles of RTVP-1 in the regulation of glioma cell proliferation were further supported by the results obtained with glioma cell overexpressing RTVP-1. Thus, overexpression of RTVP-1 induced an increase in glioma cell proliferation. Similarly, RTVP-1-overexpressing cells displayed an
increased anchorage-independent growth in soft agar. Furthermore, we found that overexpression of RTVP-1 increased the proliferation of cultured normal astrocytes. Collectively, these results suggest that RTVP-1 plays an important role in the growth of astrocytes and glioma cells.

We also found that cells overexpressing RTVP-1 by either adenovirus vectors or by stable expression exhibited an increased resistance to the apoptosis induced by serum deprivation and by TRAIL. Thus, these data further support the results of the siRNA studies that RTVP-1 regulates the survival of glioma cells. In an attempt to explore the mechanisms involved in the survival function of RTVP-1, we examined the expression and phosphorylation of various apoptosis-related proteins. We found that overexpression of RTVP-1 induced an increase in the expression of Bcl2, whereas it did not significantly alter the expression of Bax. The increase in Bcl2 partially mediated the protective effect of RTVP-1, because silencing of Bcl2 decreased the protective effect of RTVP-1 against TRAIL-induced apoptosis. The expression of Bcl2 has been associated with cell survival in various cellular systems (30), including glioma cells (31, 32). Indeed, overexpression of Bcl2 protected glioma cells from diverse apoptotic stimuli, such as FAS ligation, chemotherapeutic drugs, and radiation (33).

Overexpression of RTVP-1 also reduced the phosphorylation of JNK, whereas it did not affect the phosphorylation of AKT. JNK has been implicated in the regulation of cell apoptosis in response to various stimuli (34, 35). Therefore, the decrease in the phosphorylated form of JNK together with the increased expression of Bcl2 may render the overexpressing cells resistant to the apoptosis induced by serum-deprivation and TRAIL.

Our results regarding the role of RTVP-1 in glioma cell survival are different from those reported by Ren et al. (12, 13) for prostate cancer cells. In their studies, Ren et al. (12, 13) reported that overexpression of RTVP-1 using adenovirus vectors induced cell apoptosis in prostate cancer cells and concluded that RTVP-1 acted as a tumor suppressor gene in these cells. The reason for the different results obtained in both studies can be probably attributed to the different cellular systems that have been used. Interestingly, the expression of RTVP-1 is higher in the normal prostate compared with prostate tumors, opposite to what is found in normal brain/astrocytes and gliomas, where RTVP-1 expression is barely detected in the normal brain but is highly expressed in the malignant astrocytic tumors. Thus, RTVP-1 may act as a tumor suppressor gene in prostate cells and as a tumor promoter in gliomas and exerts opposite effects on cell apoptosis in these two cellular systems. Alternatively, the opposite effects of RTVP-1 in the two cellular systems may be due to the different processing of RTVP-1 in these cells. Based on their deletion studies, Ren et al. (12) suggested that the apoptotic effect of RTVP-1 is mediated by a secreted form of the protein. Thus, differences in the levels of cellular and extracellular forms of RTVP-1 in gliomas and prostate cancer cells may influence the apoptotic potential of RTVP-1. Additional studies using specific anti-RTVP-1 antibodies are essential to explore these possibilities.

In addition to its role in cell growth and survival, RTVP-1 also regulated the invasion of glioma cells in vitro. Thus, RTVP-1-overexpressing cells migrated more through Matrigel compared with control vector cells. In addition, we showed that RTVP-1 overexpressors exhibited stronger invasive properties into astrocytic spheroids compared with control vector cells. One of the mechanisms that contribute to the increased invasion of glioma cells is the expression and activation of MMPs (36, 37). MMPs are a family of enzymes involved in the degradation of extracellular...
matrix and therefore play a role in tumor invasion and metastasis (38, 39). We found that in control U87 cells, the active forms of MMP2 and MMP9 were undetected. In contrast, the activity of MMP2 was significantly increased in cells overexpressing RTVP-1, further suggesting that RTVP-1 increases the invasive potential of glioma cells. Our results indicate that although U87 express endogenous RTVP-1, its overexpression further enhances the growth, survival, and invasion of these cells.

The activation of MMP2 is regulated by the interaction between MMP2, MT1-MMP, and the tissue inhibitor of metalloproteinase-2 (40, 41). The mechanisms by which RTVP-1 induces the activation of MMP2 are currently under investigation. However, it is noteworthy that overexpression of Bcl2 has been recently shown to increase the activity of MMP2 via a furin-dependent pathway and to regulate the motility and invasiveness of glioma cells (42, 43). Thus, the increased expression of Bcl2 in the RTVP-1-overexpressing cells may also play a role in the increased MMP2 activity and invasion of the glioma cells in addition to its antiapoptotic effect.

In summary, we found that RTVP-1 is highly expressed in glioblastomas compared with low-grade astrocytomas and normal brains and that it plays a role in the regulation of the growth, survival, and invasion of glioma cells. Gliomas exhibit poor prognosis mainly due to their invasive potential and resistance to current therapeutic modalities. Thus, defining novel molecular targets is essential for the development of more effective therapeutic strategies. The results of this study suggest that RTVP-1 may serve as a diagnostic marker and a novel therapeutic target in the treatment of gliomas.

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