# REIC/Dkk-3 induces cell death in human malignant glioma

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**The progression of glioma to more malignant phenotypes results from the stepwise accumulation of genetic alterations and the consequent disruption of the apoptotic pathway and augmentation of survival signaling. REIC/Dkk-3, a member of the human Dickkopf (Dkk) family, plays a role as a suppressor of the growth of several human cancers; however, to date it has not been identified in brain tumors. We compared the gene and protein expression of REIC/Dkk-3 in human malignant glioma and normal brain tissues using quantitative real-time PCR, Western blotting, and immunohistochemistry. We also performed small interfering REIC/Dkk-3 (siREIC/Dkk-3) knockdown and REIC/Dkk-3 overexpression experiments to examine the role of REIC/Dkk-3 in human malignant glioma cells in vitro. In brain tissue from patients with malignant glioma, the gene and protein expression of REIC/Dkk-3 was lower than in normal brain tissue and was related to the malignancy grade. In the primary glioblastoma cell line, REIC/Dkk-3 transfection led to apoptosis owing to the activation of phosphorylated JUN, caspase-9, and caspase-3 and the reduction of β-catenin; in REIC/Dkk-3 knockdown experiments, cell growth was augmented. Our results suggest that REIC/Dkk-3 regulates the growth and survival of these cells in a caspase-dependent and -independent way via modification of the Wnt signaling pathway. Our work is the first documentation that the gene and protein expression of REIC/Dkk-3 is downregulated in human malignant glioma. Our demonstration of the mechanisms underlying REIC/Dkk-3-induced cell death indicates that REIC/Dkk-3 plays a pivotal role in the biology of human malignant glioma and suggests that REIC/Dkk-3 is a promising candidate for molecular target therapy.** *Neuro-Oncology 10, 244–253, 2008 (Posted to Neuro-Oncology [serial online], Doc. D07-00077, April 28, 2008. URL http://neuro-oncology.dukejournals.org; DOI: 10.1215/15228517-2008-016)*

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Malignant gliomas are the most common primary tumors of the brain; they are aggressive, highly invasive, destructive, and the most highly vascularized tumors in humans.[1] Despite recent progress in combination therapy, including surgery, radiotherapy, and chemotherapy, malignant gliomas remain resistant to current treatment strategies.

The WHO classifies gliomas into grades I–IV according to their histological degree of malignancy. The cumulative 5-year survivals are 66.5%, 23.4%, and 7.0% in patients with astrocytoma, anaplastic astrocytoma, and glioblastoma, respectively; the median survival in patients with the most malignant glioma, glioblastoma, is less than 1 year.[2,3]

The malignant transformation of glioma is a consequence of the stepwise accumulation of genetic alterations that lead to disruption of the apoptotic pathway and the aberrant augmentation of the survival signal.[4] Various signaling pathways, important among them the Wnt/β-catenin pathway, regulate apoptosis and survival.[5] The Wnt/β-catenin signal was originally identified as an oncogene activated by the mouse mammary tumor virus in murine breast cancer.[6] In the presence of Wnt signaling, stabilization of β-catenin stimulates cell proliferation in many biological contexts.

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The Dickkopf (Dkk) family comprises Dkk-1, -2, -3, and -4. Human Dkk-1 and -4, but not Dkk-2 and -3, inhibit Wnt signaling by binding to the transmembrane receptors Krm and LRP5/6, co-repressors of the Wnt/Fz receptor.[7–11] The expression of REIC/Dkk-3 is reduced in several human cancers; in human prostate tissue, the reduction in expression is related to the malignancy grade.[12] However, REIC/Dkk-3 has not been identified in brain tumors.[13–15]

In this study, we examined the protein and mRNA expression levels of REIC/Dkk-3 in human malignant gliomas and normal brain tissue. In addition, to investigate the biological role of REIC/Dkk-3 in malignant glioma cells, we suppressed its expression with small interfering REIC/Dkk-3 (siREIC/Dkk-3) in a U251MG malignant glioma cell line and transfected primary malignant glioma cells with the *REIC/Dkk-3* gene using a plasmid vector. We found that, in malignant glioma, *REIC/Dkk-3* expression was down-regulated and that the gene regulates cell growth through caspase-dependent apoptosis and a reduction in β-catenin.

## Materials and Methods

### *Tissue Samples*

With permission from the Ethics Committee, we obtained tissue samples from the Department of Neurosurgery of the University of Tokushima Graduate School. We studied 39 samples: 17 from glioblastomas, 11 from astrocytomas, and 11 from nonneoplastic brain tissues. One group of samples was fixed in 4% formalin in phosphate-buffered saline (PBS) and processed for paraffin embedding; the other was fresh-frozen in liquid nitrogen and stored at −70°C. All samples were classified by neuropathologists according to the WHO classification of brain tumors.

### *Cell Lines*

The human malignant glioma cell lines U251MG and GB1 were purchased from the Health Science Research Resources Bank (Osaka, Japan); U87MG and T98G were from American Type Culture Collection (Manassas, VA, USA). TGB is a primary malignant glioma cell line from a patient who granted prior informed consent for its use in this study. Normal human astrocyte (NHA) cells were purchased from Cambrex (East Rutherford, NJ, USA). All malignant glioma cell lines were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) (Gibco-BRL, Invitrogen) at 37°C in 5% $CO_2$ and air. NHA cells were grown at 37°C in 5% $CO_2$ and air in astrocyte basal medium (Cambrex) supplemented with 0.1% human recombinant epidermal growth factor, 0.25% insulin, 0.1% ascorbic acid, 0.1% gentamicin-amphotericin B mix (GA-1000), 1% L-glutamine, and 3% FBS.

### *Immunofluorescence Staining*

*Tissues.* Sections (7 μm) from formalin-fixed, paraffin-embedded tissue samples were placed on charged glass slides, deparaffinized with xylene, and rehydrated through a graded alcohol series. Tissue samples were immunostained with antihuman Dkk-3 antibody (R&D Systems, Minneapolis, MN, USA), diluted 1:100 in PBS (pH 7.4) in the presence of 1.0% bovine serum albumin (BSA), and incubated overnight in a 4°C humidity chamber. Residual antibody was washed off with PBS (3 × 5 min each), and the samples were then reacted with the secondary-antibody–labeled Alexa Fluor 488–conjugated donkey antigoat IgG antibody (Invitrogen). 4',6-Diamidino-2-phenylindole (DAPI) was used for nuclear staining.

*Cells.* REIC/Dkk-3 protein expression in the malignant glioma cell lines U251MG, GB1, U87MG, T98G, and TGB was investigated by immunofluorescence staining. Formalin-fixed cells were immunostained with polyclonal antihuman Dkk-3 antibody (R&D Systems) at a 1:100 dilution in PBS (pH 7.4) in the presence of 1.0% BSA and incubated for 1 h at room temperature. After removal of residual antibody by three 5-min washes with PBS, the cells were reacted with the secondary-antibody–labeled Alexa Fluor 488–conjugated donkey antigoat IgG antibody (Invitrogen). DAPI was used for nuclear staining.

### *Quantitative Real-Time PCR*

Using quantitative real-time PCR, we examined the expression of REIC/Dkk-3 mRNA in six glioblastoma, eight astrocytoma, and eight nonneoplastic tissue samples, in the malignant glioma cell lines U251MG, GB1, U87MG, T98G, and TGB and in NHA cells. Total RNA isolated from tumor samples, malignant glioma cell lines, and NHA cells was purified using the RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol and reverse-transcribed using the QuantiTect Reverse Transcription kit (Qiagen). Real-time PCR was performed under the conditions recommended by the manufacturer on a Light Cycler Rapid Thermal Cycler (Roche Diagnostics, Lewes, UK). The forward and reverse primer sequences were 5'-GTAAGTTCCCCTCTGGCTTG-3' and 5'-AAGCACCAGACTGTGAAGCCT-3' for REIC/Dkk-3 and 5'-GGGTGTGAACCATGAGAAGTATGA-3' and 5'-TGCTAAGCAGTTGGTGGTGC-3' for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). REIC/Dkk-3 expression was normalized with the GAPDH mRNA content.

### *Cell Index*

The cellular status was continuously monitored with the real-time cell electronic sensing (RT-CES) system, which is based on electrical-impedance measurement and is a label-free, simple homogeneous assay system for cell-based assays. The cell index number in this system

reflects the cell growth rate and cell proliferation and is comparable to actual cell-number counts and to values determined using a luminescence cell viability kit. The system comprises three components: the analyzer and E-plate station, integrated software, and a 16-well E-plate. The E-plate station, located in the incubator, is connected to the external analyzer via a thin cable. The E-plate containing the cells is placed on the E-plate station, and experimental data are collected automatically by the analyzer under the control of the integrated software, which can display the entire history of the experiment from cell seeding to cell death. The electronic readout is displayed as an arbitrary unit called the cell index, defined at each time point as (Rn – Rb)/Rb, where Rn is the cell-electrode impedance of the well containing cells and Rb is the background impedance of the well containing medium only.[16–19]

### *Western Blot Analysis*

To examine extracellular REIC/Dkk-3 expression, the culture medium and the supernatant of harvested cells before the addition of lysis buffer were collected and concentrated using a centrifugal filtering unit (Microcon YM-30; Millipore, Billerica, MA, USA), and the protein content was determined using a BCA™ Protein Assay Kit (Thermo scientific, Rockford, IL, USA) kit so that a consistent amount of protein could be taken from the different samples. Tissue samples and the precipitate from harvested cells were homogenized in lysis buffer (Cell Signaling Technology, Beverly, MA, USA). After 5-min centrifugation at 10,000 rpm, the supernatant was assayed for the protein concentration as described above. After reduction in 60 mM Tris-HCl buffer (pH 6.8) including 10% glycerol, 2% sodium dodecyl sulfate (SDS), 100 mM dithiothreitol (DTT), and 0.002% brom-phenol blue, 50 μg of protein was separated by SDS-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes (Trans-Blot Transfer Medium, BIO-RAD, Hercules, CA, USA). These were placed in blocking buffer (5% nonfat dry milk in Tris-buffered saline [TBS]) for 1 h and incubated with antihuman Dkk-3 antibody (R&D Systems) at a 1:100 dilution in Tween-TBS (T-TBS). β-Catenin antibody (BD Biosciences, San Jose, CA, USA) was diluted 1:500 in T-TBS; cleaved caspase-3, -8, and -9 antibodies (Asp175, Aso384, and Asp330, respectively), SAPK/JNK antibody, and phospho-SAPK/JNK (Thr183/Tyr185) antibody (Cell Signaling Technology) were diluted 1:1,000 in Can Get Signal solution I (Toyobo, Osaka, Japan). β-Actin antibody (Sigma Chemical Co., St. Louis, MO, USA) was diluted 1:5,000 in 5% nonfat dry milk. After three 5-min washes in T-TBS, the membranes were incubated for 1 h with the horseradish peroxidase–conjugated second antibody in T-TBS or Can Get Signal solution II at a 1:1,000 dilution. Protein-antibody complexes were detected with an enhanced chemiluminescence (ECL) system according to the manufacturer's instructions (Amersham, Piscataway, NJ, USA) on X-ray films (Amersham). To calculate the protein levels obtained by Western blot analysis, we used ImageJ 1.39 software (http://rsb.info.nih.gov/ij).

### *Transfection of siRNA and Plasmid*

Synthetic small interfering RNA (siRNA) was purchased from Qiagen. The target sequence for Dkk-3 was (sense) GGUUGUGACUCUAAGCUCA, (antisense) UGAGCUUAGAGUCACAACC. U251MG cells were transfected with siRNA to suppress the human *Dkk-3* gene using HiPerFect transfection reagent (Qiagen) according to the manufacturer's protocol.

### *Plasmid and Transfection*

Full-length human REIC/Dkk-3 cDNA was subcloned into PcDNA3.1 plasmids (generated by and purchased from TaKaRa, Mie, Japan); malignant glioma cells were plated at $3 \times 10^5$ cells/ml. After 24 h, cultures were transfected with REIC/Dkk-3 using FuGENE 6 (Roche, Basel, Switzerland) according to the manufacturer's protocol. Control cells were transfected with an empty PcDNA3.1 vector.

### *Apoptosis Detection by TUNEL and Flow Cytometric Assay*

For terminal transferase dUTP nick end labeling (TUNEL) staining with an in situ apoptosis detection kit (TaKaRa), the cells were fixed in 4% paraformaldehyde at room temperature (15–25°C, 20 min), washed 3× with PBS, and permeabilized for 5 min on ice with permeabilization buffer. After three washes with PBS, the cells were incubated (37°C, 90 min) in a TUNEL reaction mixture (labeling-safe buffer and terminal deoxynucleotidyl transferase enzyme) to label 3'-OH end DNA strand-breaks with fluorescein-dUTP. Using a fluorescent microscope, we randomly counted six fields with >100 cells/field.

Adherent and floating cells were mixed, washed with cold PBS, fixed for 30 min in 70% cold ethanol on ice, washed once with PBS, resuspended with 10 μg/ml protease-free RNase in PBS, and incubated for 20 min at room temperature (15–25°C). Then they were washed once with PBS, suspended in 1 ml PBS, and stained with 12.5 μg/ml propidium iodide (PI). At least $2 \times 10^4$ cells per sample were analyzed in an EPICS XL-MCL FACScan (Coulter Corp., Hialeah, FL, USA) using the Coulter cytological program. Experiments were performed in triplicate.

### *Statistical Analysis*

Student's *t*-test was used to compare the difference between means; $p$ values $<0.05$ were considered statistically significant.

## Results

### *Protein and mRNA Expression of REIC/Dkk-3 in Malignant Glioma Tissues*

Using immunohistochemical analysis, we found that normal brain tissue, unlike tissue from malignant

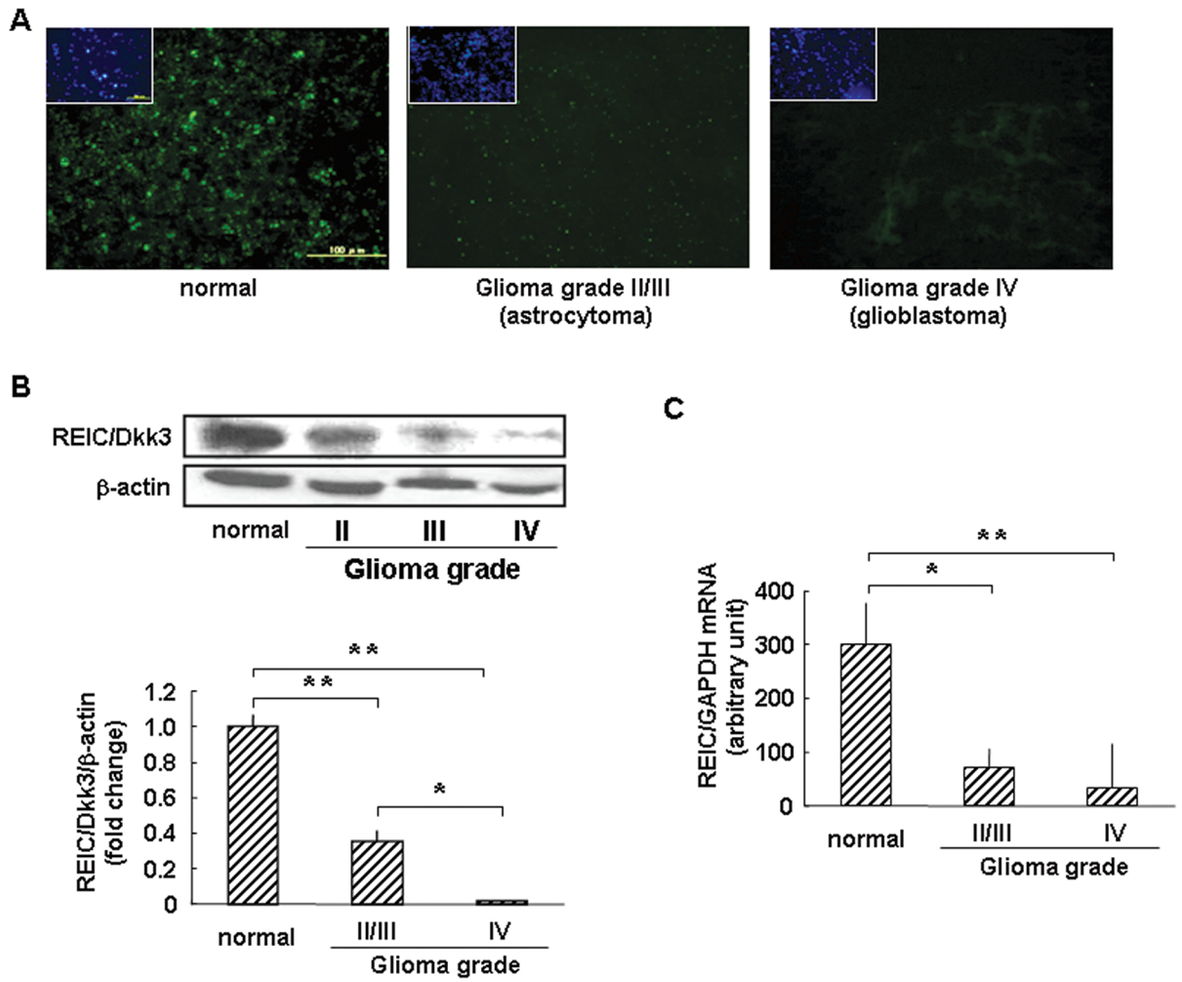


Fig. 1. Protein and mRNA expression of REIC/Dkk-3 in human brain tissue samples. (A) Immunofluorescence staining for REIC/Dkk-3 protein (green) in control temporal lobectomy specimens and astrocytoma and glioblastoma samples. The cells were counterstained for nuclear DNA (blue). The controls stained more strongly and diffusely for REIC/Dkk-3 protein than did glioblastoma, and the degree of staining was correlated with the malignancy grade. (B) The protein level of REIC/Dkk-3 was analyzed in three control lobectomy specimens, three astrocytomas (glioma grades II and III), and three glioblastomas (glioma grade IV) by Western blot analysis, and the protein band density was calculated using Image-J software. Taking the average REIC/Dkk-3 protein level in the three controls as 1.00, the average level was 0.36 in the three astrocytomas ($p < 0.05$) and 0.02 in the three glioblastomas ($p < 0.05$), indicating that the higher the WHO glioma grade, the lower the expression of REIC/Dkk-3 protein. (C) Using real-time PCR, we estimated the expression level of REIC/Dkk-3 mRNA in seven control lobectomy specimens, eight astrocytomas, and six glioblastomas. The expression level of REIC/Dkk-3 mRNA relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was significantly lower in glioblastoma than in the controls ($p < 0.01$). The decrease in REIC/Dkk-3 mRNA expression was inversely correlated with the glioma grade.

glioma, contained many diffusely distributed REIC/Dkk-3-positive cells (Fig. 1A). In normal brain tissue, most cells expressing REIC/Dkk-3 were positive for glial fibrillary acidic protein, indicating that normal glia cells express REIC/Dkk-3 (data not shown). Consistent with this observation, the protein and mRNA expression levels of REIC/Dkk-3 were lower in malignant glioma than in normal brain tissue and were inversely correlated with the malignant glioma grade ($p < 0.01$) (Fig. 1B,C).

### *Decreased mRNA and Protein Expression of REIC/Dkk-3 in Malignant Glioma Cell Lines*

Immunohistochemical analysis indicated that REIC/Dkk-3 protein was highly expressed in NHA cells and their extracellular matrix but not in U87MG or TGB cells (Fig. 2A). We then examined the expression of REIC/Dkk-3 in malignant glioma cells and culture medium including extracellular matrix using Western blot analysis. We found that the expression of REIC/Dkk-3 in the culture medium was higher from NHA and U251MG cells than from U87MG and TGB cells. Intracellular protein expression was moderately lower

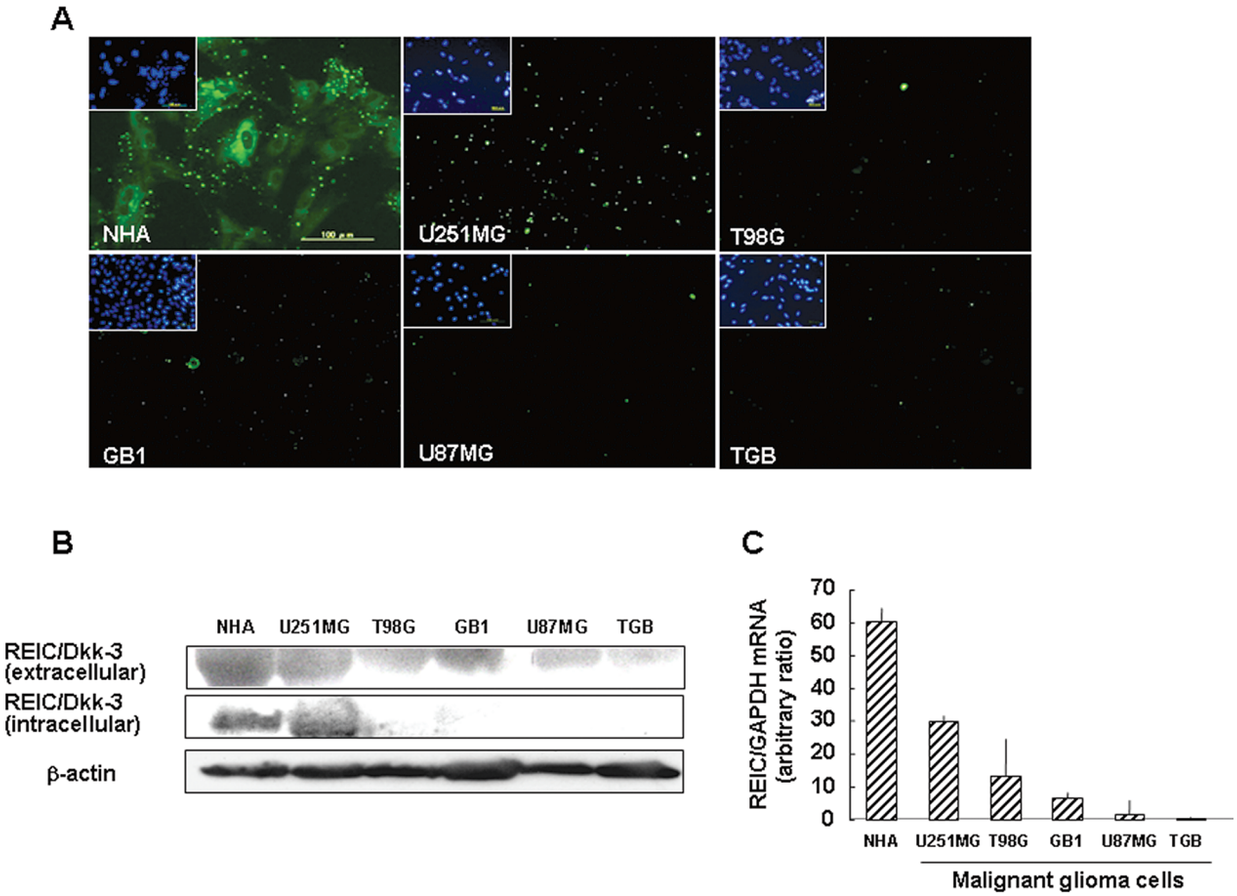


Fig. 2. Protein and mRNA expression of REIC/Dkk-3 in malignant glioma cell lines. (A) Immunofluorescence staining for REIC/Dkk-3 protein (green) in malignant glioma cell lines U251MG, T98G, GB1, U87MG, and TGB and in normal human astrocytes (NHA). NHA cells stained more strongly and diffusely than did the malignant glioma cell lines. U251MG cells stained most strongly; U87MG and TGB cells were almost stain negative. (B) Using Western blot analysis, we assessed REIC/Dkk-3 protein expression in malignant glioma cell lines U251MG, T98G, GB1, U87MG, and TGB, in NHA cells, and in culture media. Expression was high in medium from NHA and U251MG cultures. Intracellular protein expression was moderately lower in the U251MG line and dramatically lower in the other malignant glioma cell lines, compared with NHA cells. (C) REIC/Dkk-3 mRNA expression was determined in NHA cells and malignant glioma cell lines U251MG, T98G, GB1, U87MG, and TGB by real-time PCR.Relative to glyceraldehyde-3-phosphate dehydrogenase (GADPH) in NHA, expression was highest in NHA, followed by U251MG cells. TGB cells did not express REIC/Dkk-3 mRNA.

in U251MG cells and drastically lower in the other malignant glioma cell lines, compared with NHA cells (Fig. 2B). Furthermore, the mRNA expression of REIC/Dkk-3 was lower in malignant glioma than in NHA cells (Fig. 2C). Our results suggest that the protein expression of REIC/Dkk-3 is regulated by REIC/Dkk-3 mRNA expression and that REIC/Dkk-3 mRNA is unstable in malignant glioma cell lines.

### *Effect of REIC/Dkk-3 Knockdown and Overexpression on Cell Growth*

We suppressed REIC/Dkk-3 expression in U251MG cells with siREIC/Dkk-3 and overexpressed REIC/Dkk-3 with plasmid vector in TGB cells.

At 48 h after transfection, immunostaining and Western blot analysis showed decreased expression compared with the small interfering-controls (si-controls). The survival cell index for the U251MG cells treated with siREIC for 96 h was 1.5 times as high as that of the si-controls (Fig. 3A).

At 24 h after transfection, the protein expression of REIC/Dkk-3 was increased in TGB cells and the growth of REIC/Dkk-3–overexpressing malignant glioma cells was drastically inhibited (Fig. 3B). Transfection of NHA cells had no effect on their growth, although these cells overexpressed REIC/Dkk-3 protein (Fig. 3C). These findings suggest that the growth of malignant glioma cells, but not of NHA cells, is regulated by the expression level of REIC/Dkk-3.

### *Induction of Apoptosis by REIC/Dkk-3 Expression*

To clarify the mechanism(s) underlying growth inhibition in REIC/Dkk-3–overexpressing cells, we performed a flow cytometric assay 48 h after transfection. As shown

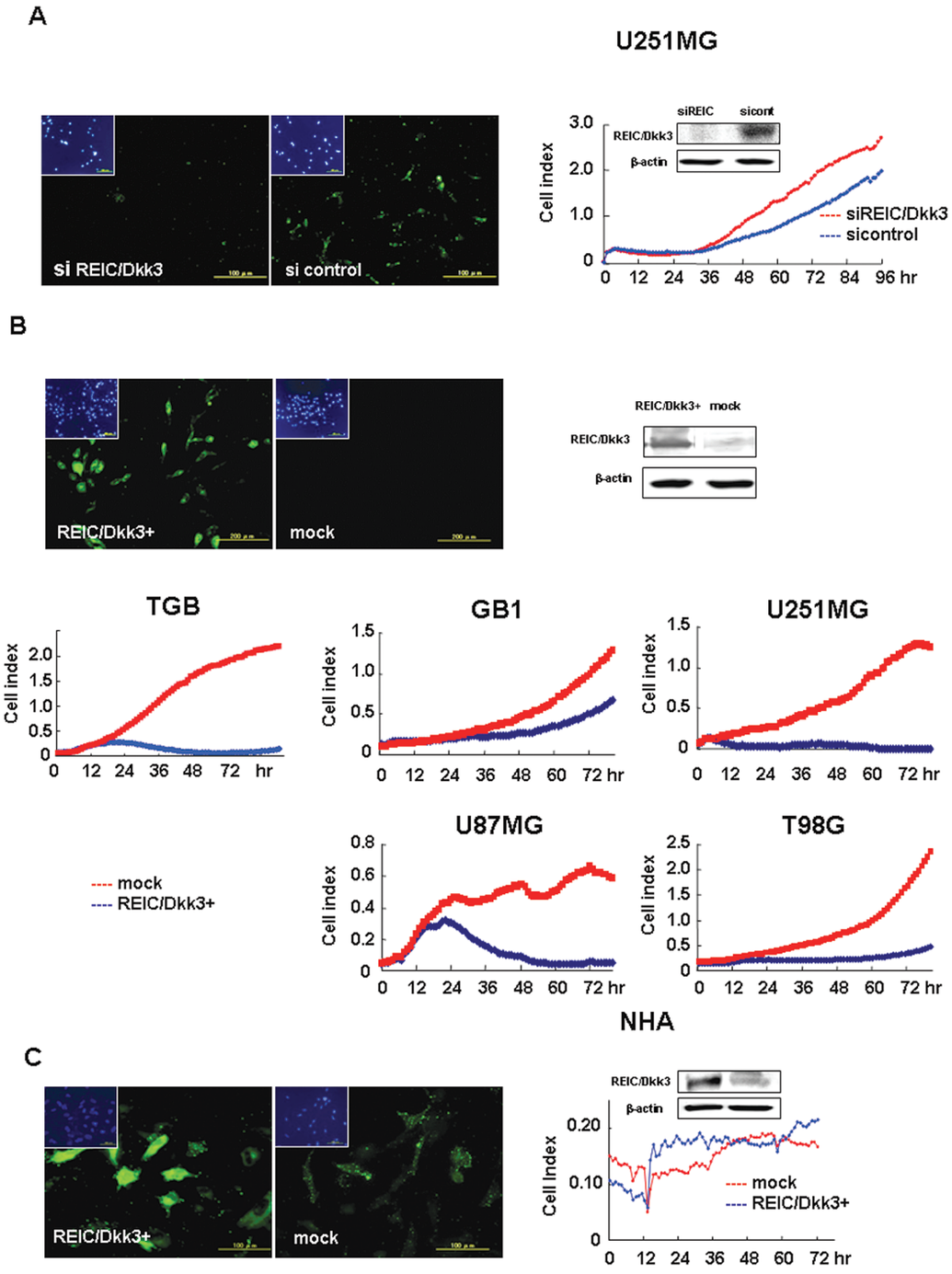


Fig. 3. Effect of REIC/Dkk-3 knockdown and overexpression on cell growth. (A) Immunofluorescence staining for REIC/Dkk-3 in U251MG cells 48 h after small interfering RNA (siRNA) transfection. The expression of REIC/Dkk-3 protein (green) in U251MG cells treated with small interfering REIC (siREIC) was lower than in the small interfering-controls (si-controls). Western blots confirmed this observation. The graph on the right shows the survival cell index of U251MG cells treated for 96 h with siREIC. Red line, siREIC; blue line, si-control. The survival cell index in siREIC-treated cells was 1.5 times as high as that in si-control. (B) Immunofluorescence staining for REIC/Dkk-3 in TGB cells at 24 h after transfection. Compared with mock-transfected cells, the transfected TGB cells were stained strongly and diffusely. Survival cell indexes of several human glioblastoma cell lines are shown in the graphs. At 72 h after transfection, all cell lines exhibited a time-dependent decrease in cell survival. Red line, mock-transfected; blue line, REIC transfected. (C) Immunofluorescence staining for REIC/Dkk-3 in NHA cells 24 h after transfection. Compared with mock-transfected NHA cells, there was strong, diffuse staining (green) in transfected normal human astrocyte (NHA) cells. Western blots showed that at 48 h after transfection, the expression of REIC/Dkk-3 protein in transfected NHA cells was significantly higher than in mock-transfected cells. On the other hand, at 72 h the growth curves of transfected and mock-transfected NHA cells were not different. Red line, mock-transfected; blue line, REIC transfected.

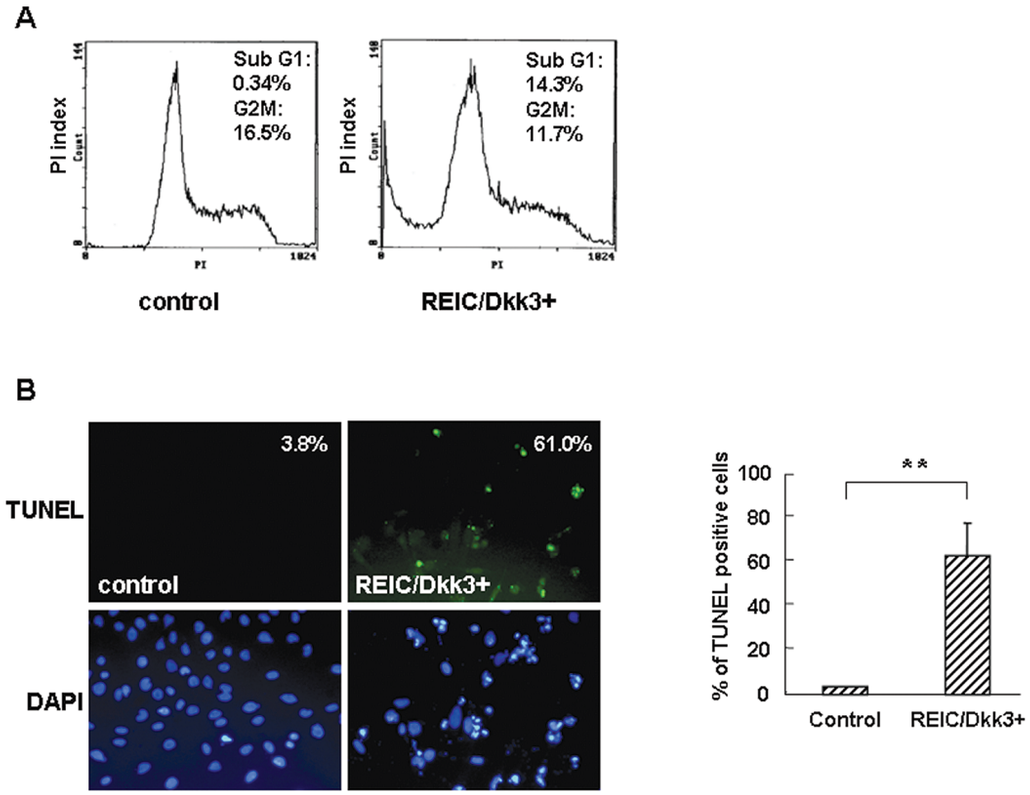


Fig. 4. Induction of apoptosis by REIC/Dkk-3 expression. (A) By flow cytometric analysis, 14.3% of transfected TGB cells and 0.34% of the controls exhibited apoptotic cells with sub-$G_1$ DNA content, indicating that REIC/Dkk-3 overexpression in the primary tumor cell line induced apoptosis. PI, propidium iodide. (B) Terminal transferase dUTP nick end labeling (TUNEL) assay performed 48 h after transfection revealed that 61.0% of transfected TGB cells were TUNEL positive, compared with 3.8% of the controls ($p < 0.01$). DAPI, 4′,6-diamidino-2-phenylindole.

in Fig. 4A, while REIC/Dkk-3 overexpression resulted in a significant ($p < 0.01$) increase in the subG$_1$ population (14.3%) compared with the controls (0.34%), there was no difference in $G_2$-M. This observation suggests that REIC/Dkk-3 promoted apoptosis rather than cell-cycle arrest.

To confirm the proapoptotic effect of REIC/Dkk-3, we performed the TUNEL assay. After transfection, 61% of the TGB cells were TUNEL positive compared with 3.8% of mock-transfected cells ($p < 0.01$) (Fig. 4B). These results confirm that REIC/Dkk-3 overexpression induced apoptosis in malignant glioma cells.

We used Western blot analysis to determine whether REIC/Dkk-3–induced apoptosis in transfected TGB cells was caspase dependent. The intra- and extracellular REIC/Dkk-3 protein levels were increased in a time-dependent manner in transfected TGB cells but not in mock-transfected cells. In the culture medium, the increase in protein expression was particularly pronounced. In TGB cells expressing REIC/Dkk-3, JNK was phosphorylated early, the expression of cleaved caspase-9 increasing parallel with the phosphorylation of JNK, and this was followed by the gradual increase in cleaved caspase-3 expression. No cleaved caspase-8 was detected (Fig. 5A). Because the activation of caspase-3 is a major apoptosis executor downstream of cytochrome C, apoptosis induction by REIC/Dkk-3 transfection may be mediated by a caspase-dependent pathway in malignant glioma cells.

### Facilitation of β-Catenin Degradation by REIC/Dkk-3 in Malignant Glioma Cells

The Wnt signaling pathway regulates cell survival, at least in part, through inhibiting the proteosomal proteolysis of β-catenin. As the role of REIC/Dkk-3 on Wnt signaling in malignant glioma cells remains unclear, we assessed its role on the expression of β-catenin in malignant glioma TGB cells. At 24 h after transfection, extracellular REIC/Dkk-3 expression was increased in an REIC/Dkk-3 expression–dependent manner (Fig. 5B). β-Catenin expression was decreased inversely with the increased expression of REIC/Dkk-3. The reduction of β-catenin was observed even when half the optimal REIC/Dkk-3 plasmid was transfected (Fig. 5B). Furthermore, the β-catenin protein level was reduced in a time-dependent manner in TGB cells treated with vector carrying REIC/Dkk-3 (Fig. 5C).

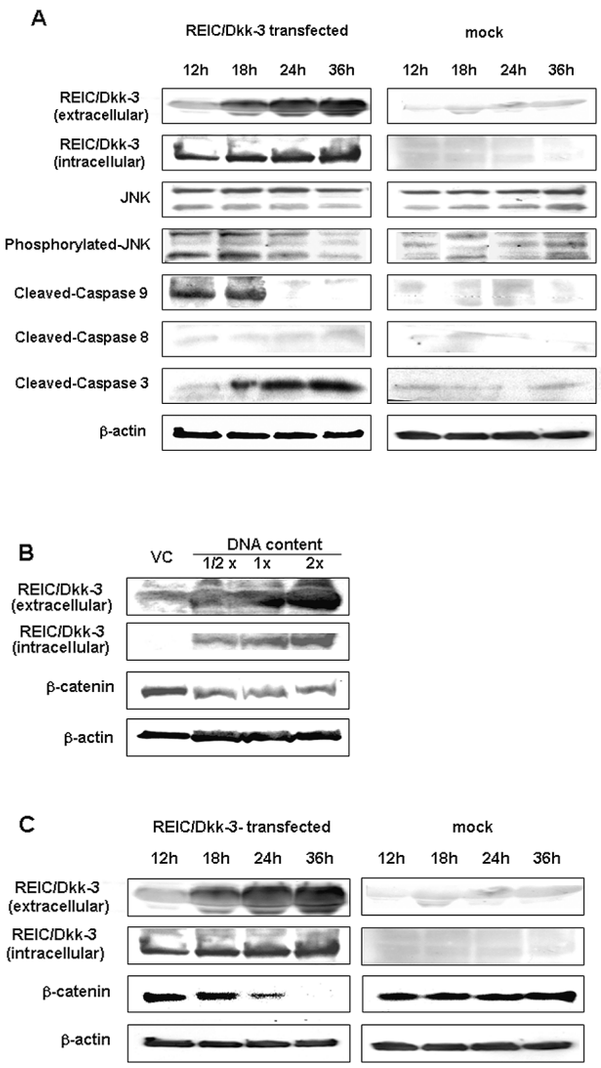


Fig. 5. Western blot analysis of REIC/Dkk-3–transfected TGB cells. (A) The REIC/Dkk-3 protein level was increased in transfected TGB cells. Especially in the culture medium, the protein level increased with time. There was no significant change in the cleaved caspase-8 level. Early in culture, REIC/Dkk-3 overexpression resulted in an increase in p-JNK and cleaved caspase-9. Caspase-3 activity increased in a time-dependent manner. (B) In transfected TGB cells, the extracellular expression of REIC/Dkk-3 was expression dependent. β-Catenin expression was decreased inversely with the increased expression of REIC/Dkk-3. The reduction of β-catenin was observed even when half the optimal REIC/Dkk-3 plasmid was transfected. (C) The extracellular expression of REIC/Dkk-3 was increased in a time-dependent manner in TGB cells treated with vector carrying *REIC/Dkk-3*. Inversely correlated with the expression of REIC/Dkk-3, β-catenin protein decreased in a time-dependent manner.

## Discussion

### *Proapoptotic Function of REIC/Dkk-3 in Malignant Glioma Cells*

*REIC/Dkk-3* is one of the down-regulated genes, not only in a variety of human immortalized- and tumor-derived cell lines, but also in human cancer tissues.[12–15,20] However, the molecular mechanisms underlying the tumor-suppressing function of REIC/Dkk-3 in human malignant glioma remain to be elucidated.

Our study is the first to demonstrate that *REIC/Dkk-3* is down regulated in human malignant glioma and that there is an inverse correlation between the expression level of REIC/Dkk-3 protein and mRNA and the malignancy grade (Fig. 1). In fact, the expression of *REIC/Dkk-3* was suppressed to undetectable levels in glioblastoma (WHO grade IV) and was lower in malignant glioma cell lines than in NHA cells (Fig. 2). Our findings suggest that a decrease in the level of REIC/Dkk-3 mRNA expression may contribute to the tumorigenesis of malignant gliomas.

As for the function of REIC/Dkk-3 in malignant glioma cells, REIC/Dkk-3 knockdown by RNA interference increased the survival cell index in U251MG cells (Fig. 3A), while its overexpression significantly decreased the survival cell index in TGB and other malignant glioma cells (Fig. 3B).

In TGB cells, increased REIC/Dkk-3 expression led to a remarkable increase in the sub$G_1$ population but not the $G_2$-M population and in the number of TUNEL-positive cells (Fig. 4). Furthermore, caspases-9 and -3, but not caspase-8, were activated by REIC/Dkk-3 (Fig. 5A). Therefore, the antiproliferative property of REIC/Dkk-3 was due to the induction of caspase-dependent apoptosis rather than to cell-cycle arrest. Our findings indicate that *REIC/Dkk-3* is one of the important genes regulating caspase-dependent apoptosis in human malignant gliomas but not NHA cells.

### *Mechanisms Underlying REIC/Dkk-3–induced Apoptosis*

Although the regulatory mechanisms of JNK by REIC/Dkk-3 remain to be elucidated, REIC/Dkk-3 induced apoptosis in prostate cancer cells via JNK activation but had no effect on β-catenin.[12] In malignant glioma, REIC/Dkk-3 activated JNK; however, the inhibition of JNK did not suppress REIC/Dkk-3–induced apoptosis (data not shown). These data suggest that the signaling pathway mediating REIC/Dkk-3–induced apoptosis in malignant glioma cells may differ from that in prostate cancer cells and that, in the malignant glioma cell lines, JNK does not play a crucial role in REIC/Dkk-3–induced apoptosis.

The activation of Wnt signaling pathways is thought to contribute to the development of some human cancers, and β-catenin is considered a key element in the Wnt signaling pathway. In the presence of Wnt signaling, glycogen synthase kinase-3β (GSK-3) is inactivated;

this leads to the accumulation and nuclear translocation of β-catenin and results in the formation of a complex with the lymphocyte enhancer factor (LEF)/T-cell factor (TCF) family of transcription factors. The transcription factor complex activates a variety of target genes involved in growth, development, and oncogenesis.[21–25] Moreover, Wnt signaling inhibits the release of cytochrome C and the subsequent activation of caspase-9 induced by apoptotic stimuli.[26] We documented the increased expression of cleaved caspase-9 and the reduction of β-catenin in parallel with the increased expression of intra- and extracellular REIC/Dkk-3 (Fig. 5B). Several previous studies have shown that Dkk-1 and -4 inhibited the Wnt signaling pathway by binding to the transmembrane receptor Krm and LRP5/6, a co-repressor of the Wnt/Fz receptor.[7–11] Furthermore, in cells exposed to recombinant Dkk-1, Wnt signaling is inhibited.[27] As is the case for Dkk-1 and -4, REIC/Dkk-3 exists predominantly extracellularly (Fig. 2), and its overexpression facilitates β-catenin degradation (Fig. 5C). Our observations suggest that extracellular REIC/Dkk-3 may be associated with REIC/Dkk-3–induced apoptosis via Wnt signaling inhibition. In the absence of Wnt signaling, β-catenin is associated with a cytoplasmic complex containing GSK-3, axin, and adenomatous polyposis coli protein (APC). In this complex, GSK-3 constitutively phosphorylates the β-catenin protein at serine and threonine residues, and phosphorylated β-catenin becomes a target for ubiquitination and subsequent degradation by the proteasome. In addition to proteasomal proteolysis, β-catenin is proteolytically cleaved by activated caspase-3 during apoptosis, resulting in some fragments being detected by Western blot analysis with the antibodies against the β-catenin we used in our study.[28] We found that the degradation of β-catenin upon REIC/Dkk-3 expression preceded caspase-3 activation, and we did not detect bands smaller than full-length β-catenin. Therefore, the degradation of β-catenin may not be a consequence of caspase-3 activation but rather might be due to inhibition of Wnt signaling via binding to the transmembrane receptors of increased extracellular REIC/Dkk-3. In our study, transfection of the *REIC/Dkk-3* gene into glioblastoma multiforme cells induced an increase in intra- and extracellular REIC/Dkk-3 protein. In parallel with this increase, caspase-3 activation was increased via caspase-9 activation, but not caspase-8 activation. These data suggest that REIC/Dkk-3 activates the intrinsic but not the extrinsic apoptosis pathway.

In prostate cancer cells, REIC/Dkk-3–induced apoptosis had no effect on β-catenin[12]; in osteosarcoma, β-catenin was redistributed, and apoptosis was not induced by REIC/Dkk-3 overexpression.[29] These findings support our hypothesis that, in malignant glioma, REIC/Dkk-3 induces caspase-dependent and -independent apoptosis and that it may exert anticancer activity at different action points in several human cancer cells. Our findings provide new insights into the role of REIC/Dkk-3.

We first documented that REIC/Dkk-3 plays a pivotal role in regulating cell survival in human malignant glioma and that it promotes caspase-dependent apoptosis and facilitates the degradation of β-catenin. The functional disruption of *REIC/Dkk-3* or its down-regulation appears to be a critical event in tumor development and progression. Thus, REIC/Dkk-3 may be a promising candidate for molecular target therapy and an outcome predictor in patients with malignant glioma.

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