

Laboratory Investigation

GRIM-19 Expression and Function in Human Gliomas

Yong-Hao Jin, M.D., Ph.D.,^{1,2,3} Shin Jung, M.D., Ph.D.,^{1,2} Shu-Guang Jin, M.D.,^{1,2} Tae-Young Jung, M.D., Ph.D.,^{1,2}
Kyung-Sub Moon, M.D.,^{1,2} In-Young Kim, M.D., Ph.D.,^{1,2}

Department of Neurosurgery & Brain Tumor Research Laboratory,¹ Chonnam National University Hwasun Hospital & Medical School, Gwangju, Korea

Department of Neurosurgery,² Chonnam National University Research Institute of Medical Sciences, Gwangju, Korea

Department of Neurosurgery,³ Yanbian University Hospital, Yanji City, Jilin Prov., China

Objective : We determined whether the expression of GRIM-19 is correlated with pathologic types and malignant grades in gliomas, and determined the function of GRIM-19 in human gliomas.

Methods : Tumor tissues were isolated and frozen at -80°C just after surgery. The tissues consisted of normal brain tissue (4), astrocytomas (2), anaplastic astrocytomas (2), oligodendrogliomas (13), anaplastic oligodendrogliomas (11), and glioblastomas (16). To profile tumor-related genes, we applied RNA differential display using a Genefishing™ DEG kit, and validated the tumor-related genes by reverse transcription polymerase chain reaction (RT-PCR). A human glioblastoma cell line (U343MG-A) was used for the GRIM-19 functional studies. The morphologic and cytoskeletal changes were examined via light and confocal microscopy. The migratory and invasive abilities were investigated by the simple scratch technique and Matrigel assay. The antiproliferative activity was determined by thiazolyl blue Tetrazolium bromide (MTT) assay and FACS analysis.

Results : Based on RT-PCR analysis, the expression of GRIM-19 was higher in astrocytic tumors than oligodendroglial tumors. The expression of GRIM-19 was higher in high-grade tumors than low-grade tumors or normal brain tissue; glioblastomas showed the highest expression. After transfection of GRIM-19 into U343MG-A, the morphology of the sense-transfection cells became larger and more spindly. The antisense-transfection cells became smaller and rounder compared with wild type U343MG-A. The MTT assay showed that the sense-transfection cells were more sensitive to the combination of interferon- β and retinoic acid than U343MG-A cells or antisense-transfection cells; the anti-proliferative activity was related to apoptosis.

Conclusion : GRIM-19 may be one of the gene profiles which regulate cell death via apoptosis in human gliomas.

KEY WORDS : Cell line · GRIM-19 · Gene Fishing · Glioblastoma · Human glioma.

INTRODUCTION

Gliomas are the most common primary tumors of the brain and more than one-half of all gliomas exhibit aggressive and malignant behavior. Glioblastoma multiforme (GBM) is clinically and pathologically the most malignant type⁴². The median survival after diagnosis is approximately 11-12 months^{27,42}. Many genetic alterations have been detected in this tumor type, and the most common genetic alterations are the tumor suppressor genes TP³⁹, the cellular

oncogene MDM2⁴⁵, and the epidermal growth factor receptor (EGFR)¹⁴. The candidates for tumor suppressor genes are cell-cycle regulators²⁰, cellular signaling proteins⁴⁷, apoptosis regulators¹⁵, and transcriptional regulators⁴⁴. However, the mechanisms underlying genetic changes in gliomas are not completely understood.

In this study, expression of several genes relating to tumor malignancy in astrocytic tumors was detected using Genefishing technique. The genes included genes associated with retinoid-IFN-induced mortality (GRIM)-19, KIAA0892, IGSF 4, protein phosphatase 1, and FLJ 21967; GRIM-19 was the gene which was most differently expressed.

GRIM-19 is known to encode a 16-kDa protein of 144 amino acids, and is one of several GRIM², a cell death regulatory protein, which is essential for the assembly and function of complex I of the mitochondrial respiratory chain¹⁹. GRIM-

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• Address for reprints : Shin Jung, M.D., Ph.D.

Department of Neurosurgery, Chonnam National University Hwasun Hospital, 160 Ilsim-ri, Hwasun-eup, Hwasun 519-809, Korea

Tel : +82-61-379-7666, Fax : +82-61-379-7673

E-mail : sjung@chonnam.ac.kr

19 also induces apoptosis in a number of cell lines upon treatment with interferon-beta (INF- β) and retinoic acid²⁾. GRIM-19-associated pathology has been reported in Hürthle cell tumors of the thyroid³⁶⁾ and renal cell carcinomas¹⁾, but GRIM-19-associated pathology have not been reported in brain tumors.

In this study, we determined whether the expression of GRIM-19 is correlated with gliomas malignancy and the action of GRIM-19 in human glioblastoma cell lines.

MATERIALS AND METHODS

RNA isolation

Total ribonucleic acid (RNA) was isolated from normal brain tissue or glioma samples using TRIZOL (Life Technologies, Gaithersburg, MD, USA), according to the manufacturer's instructions. After isolating the RNA, the RNA pellets were eluted with RNase-free water and stored at -80°C until use.

Differentially display-polymerase chain reaction

Differentially display-polymerase chain reaction (DD-PCR) was performed using a Genefishing™ kit (Seegene Inc., Seoul, Korea), according to the manufacturer's instructions. For first strand synthesis, 3 μ g of the purified total RNA was incubated with 10 μ M dT-ACP (1 μ L; the volume of the μ L of 5 \times RT buffer (Promega, Madison, WI, USA), dNTP (2 mM each), 2.5 μ L of 25 mM MgCl₂, 2 μ L of RNase inhibitor (40 U/ μ L; Promega), and 1 μ L of reverse transcriptase (200 U/ μ L; Promega) were added. The volume of the reaction mixture was 20 μ L, and it was allowed to react for 60 minutes at 42°C, then for 15 minutes at 70°C. The polymerase chain reaction (PCR) protocol for the second-strand synthesis was one cycle at 94°C for 3 minutes, followed by 50°C for 3 minutes, and 72°C for 1 minute. After the second-strand DNA synthesis was completed, the second-stage PCR amplification protocol was 40 cycles at 94°C for 40 seconds, 65°C for 40 seconds, 72°C for 40 seconds, and this was followed by a 5 minutes final extension step at 72°C. The PCR products were separated in 1.2% agarose gel, and stained with ethidium bromide. The differentially expressed bands were extracted from the gel using a QIAquick Gel extraction kit (Qiagen, Carlsbad, CA, USA), and were directly cloned into pGEM-T Easy vector (Promega) without re-amplification of the recovered bands, and then sequenced.

Reverse transcription polymerase chain reaction

One μ g of RNA was reverse transcribed to synthesize the complementary deoxyribonucleic acid (cDNA). The reverse transcription (RT) was followed by PCR. For the first strand

synthesis, 1 μ g of the purified total RNA was incubated with oligo dT (0.5 μ g/ μ L, Promega) for 5 minutes at 70°C, then a buffer solution containing 4 μ L of 5X reaction buffer (Promega), 1 μ L of dNTP (10 mM each), 3.5 μ L of 25 mM MgCl₂, 2 μ L of RNase Inhibitor (40 U/ μ L; Promega), and 1 μ L of reverse transcriptase (200 U/ μ L; Promega) was added. The reaction volume was 20 μ L and the reaction was performed for 90 minutes at 42°C, then for 2 minutes at 90°C. The PCR primers were designed to amplify the 553 bp fragment of the GRIM-19 open reading frame, as follows : forward primer, 5'-TGCAAGAACCAAGGCGAGTCAC-3'; and reverse primer, 5'-GGCACGTCCTTCATGATGATGG-3'. GAPDH was used as an internal control. The primers were designed to amplify the 114 bp fragment, as follows : forward primer, 5'-GTTGAGG TCAATGAAGGG-3'; and reverse primer, 5'-GTGAAGGT CGGAGTCAAC-3'.

Preparation of the plasmid containing human GRIM-19 cDNA and plasmid construction

The cDNA synthesized from the total RNA was used as a template for the PCR reaction. The PCR primers were designed to amplify the full-length human GRIM-19-sense (GRIM-19-S) cDNA open reading frame, as follows : forward primer, 5'-CGGGATCCATGCAAGAACCAAGGC GAG-3'; and reverse primer, 5'-GCTCTAGAGCCTACG TGTACCACATGAAGC-3'. The BamHI (GGATCC) sites were engineered into the forward PCR primer, and the XbaI (TCTAGA) sites were engineered into the reverse primer. The GRIM-19-antisense (GRIM-19-AS) PCR primers open reading frame were as follows : forward primer, 5'-GCTCTAGAGCATGCAAGAACCAA GGCGAG-3'; and reverse primer, 5'-CGGGATCCCTACGTGTACCAC ATGAAGC-3'. The XbaI (TCTAGA) sites were engineered into the forward primer, and the BamHI (GGATCC) sites were engineered into the reverse primer. The PCR products were separated by electrophoresis through 1% agarose gel and the expressed bands were extracted from the gel using a QIAquick Gel extraction kit (Qiagen) and subcloned into pGEM-T Easy Vector (Promega), according to the manufacturer's instructions, and then sequenced.

The full-length human GRIM-19 cDNAs were cloned in the sense and antisense orientations into the BamHI and XbaI sites of an expression plasmid vector [pcDNA3.1(+/-)] containing the CMV promoter and the neomycin-resistance gene. The resulting vectors, pcDNA3.1(+/-)-GRIM-19-S and pcDNA3.1(+/-)-GRIM-19-AS, were used to transfect the plasmid DNA.

Cell lines and cell culture

U343MG-A cells were obtained from the Brain Tumor

Research Center (University of California, San Francisco, CA, USA). The cell lines were routinely grown in DMEM (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified 95% air/5% CO₂ atmosphere.

Transfections

The malignant glioma cell line, U343MG-A, was maintained under exponential growth conditions in DMEM supplemented with 10% FBS in the absence of antibiotics. The optimum cell density for transfection is normally between 50% and 80% confluence for adherent cells. pcDNA3.1(+/-)-GRIM-19-S and pcDNA3.1(+/-)-GRIM-19-AS were trans-fected into U343MG-A cells using the GeneJuice transfection reagent (1.33 mg/mL suspension in 80-90% ethanol; Novagen Corp, USA). These transfectants are referred to herein as U343-G-S and U343-G-AS, respectively. The cells in serum-free DMEM were mixed with 10 µg of plasmid DNA and 30 µL of GeneJuice/serum-free media according to the manufacturer's protocol. After 5 hours of incubation at 37°C, the transfection mixture was replaced with DMEM supplemented with 10% FBS. After 24 hours of incubation, the medium was replaced with DMEM containing 10% FBS and 800 µg/mL of G418, and cultured in a CO₂ incubator. The G418-resistant clones were isolated, and the level of expression of GRIM-19 protein was determined by Western blotting analysis and immunofluorescence staining. The stable transfectants were maintained in DMEM supplemented with 10% FBS and 400 µg/mL of G418.

Western Blotting

Cells were lysed in a lysis buffer [50 mM Tris (pH 8.0), 5 mM EDTA, 150 mM sodium chloride, 0.5% deoxycholic acid, 0.1% SDS, 1% NP-40, 1 mM PMSF, and 1 mg/mL protease inhibitor cocktail]. The protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Fifty µg of the whole cell lysates were separated by 15% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Pall Corporation, USA). Subsequently, the membrane was incubated for 2 hours at room temperature in a solution of TBST [10 mM TrisCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20] supplemented with 5% non-fat dry milk, and probed overnight at 4°C with anti-GRIM-19 (1 : 1,000; BD Transduction Laboratories, USA). The bound antibodies were visualized with goat anti-mouse antibody (1 : 80,000; Jackson ImmunoResearch, West Grove, PA, USA) conjugated with horseradish peroxidase using enhanced chemiluminescence reagents (ECL, Amersham Biosciences, USA).

Immunofluorescence staining and immunocytochemistry of transfectant

In the immunofluorescence staining, cells were cultured on coverslips in 35-mm dishes until subconfluence, washed with phosphate buffer saline (PBS), and fixed with 4% paraformaldehyde for 10 minutes. After washing (3-5 times) in immuno/DNA buffer (Invitrogen Corporation, USA), the cells were treated with 0.1% Triton X-100 for 5 minutes at room temperature and washed 3-5 times. The cells were incubated with anti-GRIM-19 antibody (1 : 500) in a humidified chamber for 1 hour, then with Alexa 488-conjugated goat anti-mouse antibody (1 : 400; Molecular Probes) for 40 minutes. The coverslips were mounted on slides with Immuno-mounts (Shandon, USA). To determine the expression of GRIM-19 protein in these cells, the fluorescence intensities of the U343-G-S, U343-G-AS, and U343MG-A cells were measured by fluorescence staining, and digitally photographed for evaluation and recording. For observation of the cellular location, the fluorescence intensities of the U343-G-S cells were measured by confocal microscopy using an Axiovert 100M confocal microscope (Carl Zeiss Micro Imaging, Inc, Germany) equipped with a Plan-Apochromat 63x/1.40 oil objective. The confocal images were acquired using LSM 510 2.3 software.

In the cytoskeleton analysis, cells were also cultured on coverslips, washed with PBS, and fixed with 4% paraformaldehyde. After washing in immuno/DNA buffer, the cells were treated with 0.1% Triton X-100. The cells were incubated with anti-vimentin (1 : 100; BD Pharmingen, USA) in a humidified chamber for 1 hour, and then with Alexa 488-conjugated goat anti-mouse antibody (1 : 400; Molecular Probes) for 40 minutes, and rhodamine-conjugated phalloidin (Molecular Probes) was used in actin staining. Finally, the coverslips were mounted onto the slides, and observation with confocal microscopy was performed.

Cell morphologic characteristics

The cells were plated in 60 mm dishes and fixed with methanol at 4°C for 5 minutes. After washing twice with PBS and staining with 0.1% toluidine blue, the cells were examined via light microscopy (Nikon, Garden City, NY, USA), and digitally photographed to evaluate and record the morphology of the cell population.

Migration test

In the migration assay, a simple scratch technique was used. To compare the motility of each cell line, 5 mM hydroxyurea was added to the culturing media. Twenty-four hours of treatment resulted in the complete inhibition of cell proliferation. Then, the cultures were scraped with a single-edged

razor blade. The cells were washed twice with PBS and cultured in medium containing hydroxyurea. After 3 days of incubation, the cells were washed twice with PBS, fixed with methanol, and stained with 0.1% toluidine blue. Three microscopic fields were evaluated for each wound injury. The number of cells migrating across the wound edge was counted in each field and averaged for each injury. The experiments were repeated three times.

Matrigel invasion assay

The invasion assay was performed using a transwell chamber and 8 mm pore size polycarbonate membranes (Costar, Cambridge, MA, USA) coated with serum-free DMEM diluted ECM (Matrigel, DMEM to ECM 3 : 1; Becton Dickson, Bedford, MA, USA). The cells were seeded at a density of 3×10^4 cells in 350 μ L of serum-free DMEM in the upper compartment of the transwell chamber, and allowed to invade the polycarbonate membrane for 48 hours. The lower chamber was filled with DMEM that contained 10% FBS. The non-invaded cells on the upper surface of the membrane were removed from the chamber, and the invaded cells on the lower surface of the membrane were stained with Diff-Quick (Kokusai Shiaku, Kobe, Japan). The numbers of invaded cells in four randomly selected microscopic fields (40 \times) per membrane were counted. These experiments were repeated three times.

Thiazolyl blue tetrazolium bromide (MTT) assay

Cells (5,000/well) were seeded in a 96-well plate and allowed to grow for 24 hours before being stimulated with IFN- β (human interferon- β , Sigma-Aldrich, Inc., St. Louis, MO, USA) and all trans retinoic acid (RA; Sigma-Aldrich, Inc). After 24 hours, combinations of INF and RA at different concentrations were added to the wells. After 24 hours, MTT (5 mg/mL) was added to each well and incubated at 37°C for 4 hours in the dark. After removing the media and MTT, 100 μ L of dimethylsulfoxide (DMSO) was added, and the plate was shaken for 15 minutes at room temperature in the dark. The absorbance was monitored at 560 nm using a Biotech ELISA plate reader.

FACS analysis

U343-G-S, U343-G-AS, and U343MG-A cell lines (3×10^5 cells each) were seeded in 30-mm culture dishes and allowed to grow for 24 hours before being stimulated by a combination of IFN- β and RA. After 24 hours, the cells were treated with a combination of IFN- β (1,500 U) and RA (2 μ M) and FACS analysis was performed at different time points after staining with an Annexin V-FITC apoptosis detection KIT I (BD Biosciences Pharmingen, San Diego, CA,

USA). The untreated cells were regarded as a negative control.

Data analysis

Comparison of the nucleotide sequence homology of the isolated cDNAs with the registered sequence in Genbank was done with the BLAST algorithm. We measured the statistical significance of the cell number and cell percentage using Student's t-test (two-tailed). *p* values < 0.05 were considered statistically significant.

RESULTS

Identification of the differentially expressed genes

To obtain the tumor-related genes, we performed DD-PCR with various astrocytic tumor samples. The arrowheads in Fig. 1 indicate the bands of the differentially expressed genes. The DD-PCR fragments were cut from the gel and ligated into the pGEM-T easy TA cloning vector system (Promega). The plasmids were sequenced and a homology search was performed in the GenBank using the BLAST program available on the National Center for Biotechnology server on the World Wide Web. The genes were identified as GRIM-19, KIAA0892, IGSF 4, protein phosphatase 1, and FLJ 21967.

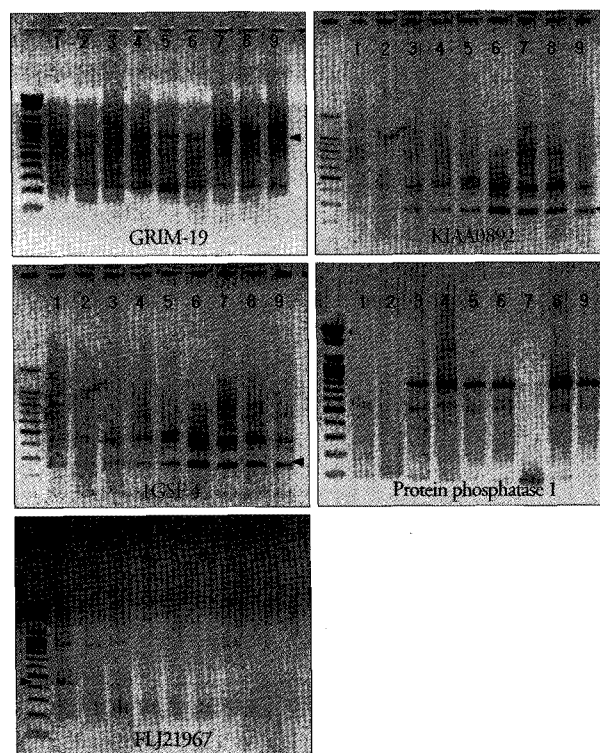


Fig. 1. Differential display (DD)-PCR for detection of tumor-related genes shows five candidates, including GRIM-19 (lane 1 : normal brain; lanes 2 and 3 : astrocytoma samples; lanes 4 and 5 : anaplastic astrocytoma samples; and lanes 6-9 : glioblastoma samples).

Validation of GRIM-19 DD-PCR data by RT-PCR

For validation of the GRIM-19 DD-PCR data, we divided the samples into two groups (the astrocytic and oligodendroglial tumor groups). The RT-PCR results showed that the expression of GRIM-19 in the astrocytic tumor group was higher than the oligodendroglial tumor group. The expression of GRIM-19 increased with increasing tumor grade. In 75% (3 of 4) of the astrocytoma and anaplastic astrocytoma groups, the expression of GRIM-19 was higher than the normal group, and the highest expression was observed in 62.5% (10 of 16) of the glioblastoma group. In the oligodendroglial tumor group, there was no significant difference between the normal and tumor groups (Fig. 2).

Establishment of a cell line with human glioma U343MG-A cells

To assess the relationship between GRIM-19 expression and the glioma cell proliferation/migration/invasion/apoptotic effect, sense or antisense GRIM-19 transfected to human glioma U343MG-A cells with a plasmid DNA that produces sense and antisense mRNA under the control of the CMV

promoter, were used to obtain neomycin-resistance transfected clones. To determine whether or not sense GRIM-19 mRNA promotes the production of GRIM-19 protein, and whether or not antisense GRIM-19 mRNA interferes with the production of GRIM-19 protein, the respective clones were selected. Monomeric GRIM-19 has a molecular weight of 16kDa. GRIM-19 was more highly expressed in the U343-G-S (sense GRIM-19 transfected U343MG-A) and less highly expressed in the U343-G-AS (antisense GRIM-19 transfected U343MG-A) compared with U343MG-A, as determined using Western blot analysis.

Immunofluorescence staining and immunocytochemistry of transfectants

In agreement with the results obtained from the Western blot analysis, immunofluorescence staining in conjunction with fluorescence microscopy revealed that GRIM-19 was highly expressed in U343-G-S and less expressed in U343-G-AS, as compared with U343MG-A (Fig. 3). In U343-G-S, GRIM-19 was expressed both in the nucleus and cytoplasm, as determined using fluorescence confocal microscopy (Fig. 4).

Immunofluorescence staining performed with the actin and vimentin cytoskeletons to determine whether or not the differences were associated with the cytoskeletal alterations and correlated with tumor cell motility and invasion (Fig. 5). U343-G-S and U343-G-AS showed no significant changes in the lamellipoda, stress fibers, or vimentin filaments compared with U343MG-A.

Morphologic characteristics of transfectants

The morphology of U343-G-S was larger and more spindly compared with U343MG-A. In the case of U343-G-AS, the morphology was smaller and

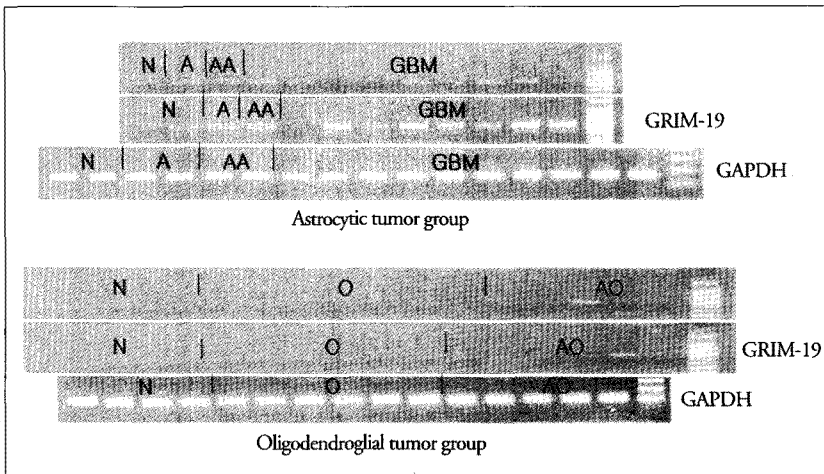


Fig. 2. Reverse transcription (RT)-PCR for GRIM-19 shows the coincidence with DD-PCR data. N : normal brain, A : astrocytoma sample, AA : anaplastic astrocytoma sample, GBM : glioblastoma sample, O : oligodendroglioma sample, AO : anaplastic oligodendroglioma.

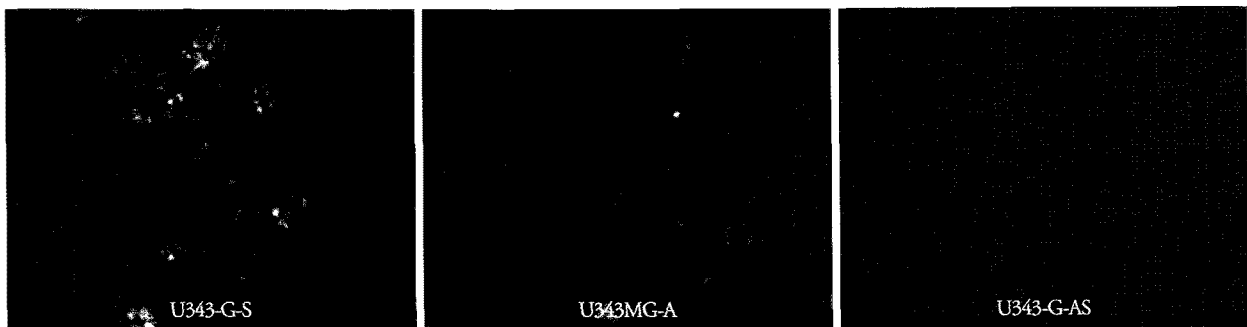


Fig. 3. The fluorescence microscopic pictures show that GRIM-19 is highly expressed in U343-G-S and less expressed in U343-G-AS, as compared with U343MG-A (original magnification × 100).

rounder compared with U343MG-A (Fig. 6).

Effects mediated by GRIM-19 on cell migration, invasion and proliferation

In the simple scratch test, the U343-G-S appeared to have more motility in terms of the distance of migration compared with U343MG-A and U343-G-AS, but there was no significant difference in the cell number ($p > 0.05$) (Fig. 7). The mean cell numbers were 76.7 ± 9.7 for U343-G-S, 84.3 ± 13.5 for U343MG-A, and 82.7 ± 10.1 for U343-G-AS.

In the Matrigel invasion assay, the mean number of invading cells was 110.3 ± 12.4 , 122.5 ± 16.1 , and 117.3 ± 14.6 for U343-G-S, U343MG-A, and U343-G-AS, respectively. There was no significant difference between the three cells ($p > 0.05$) (Fig. 8).

The MTT assay was performed to investigate whether GRIM-19 associated with the combination of INF and RA regulates cell survival. To select the best concentration by carrying out the next experiment, U343-G-S cells were treated with combinations of INF and RA at various concentrations for 24 hours. In the U343-G-S cells, the anti-proliferative activity increased with increasing concentrations of INF/RA. The survival rate was 74.1% when the concentration of INF was 1,500 U and the concentration of RA was 2 μM (Fig. 9A). We selected this concentration to perform the next experiment. To observe if the anti-proliferative activity of GRIM-19 was enhanced, we treated U343-G-S cells with INF/RA, INF, and RA for 24 hours. In the MTT assay, the anti-proliferative activities of GRIM-19 treated with INF, RA, and INF/RA were 6.6%, 7.9%, and 24.2%, respectively (Fig. 9B). This suggested that the combination of INF and RA enhanced the anti-proliferative activity. After being treated with INF/RA for 24 hours, the survival rates of the U343-G-S, U343MG-A, and U343-G-AS cells were 74.1%, 81.1%, and 97%, respectively (Fig. 9C). Compared with the U343MG-A and U343-G-AS cells, the U343-G-S cells were more sensitive to the combination of IFN and RA in terms of the anti-proliferative activity.

FACS analysis was performed to investigate whether the high anti-proliferative activity observed in the MTT

assay was caused by apoptosis. After being treated with INF/RA for 24 hours, the death rate of U343-G-S, U343MG-A, and U343-G-AS was 24.5%, 12%, and 7.9%, respectively (Fig. 10A). By observing the cell cycle, we detected cell death

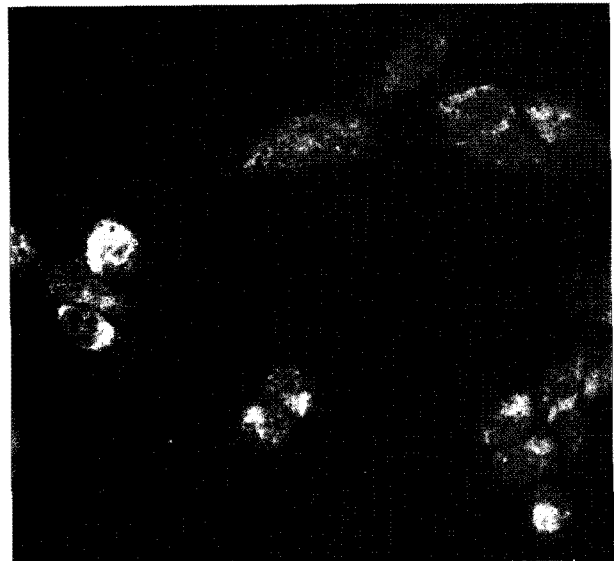


Fig. 4. GRIM-19 is expressed both in the nucleus and cytoplasm in U343-G-S using fluorescence confocal microscopy.

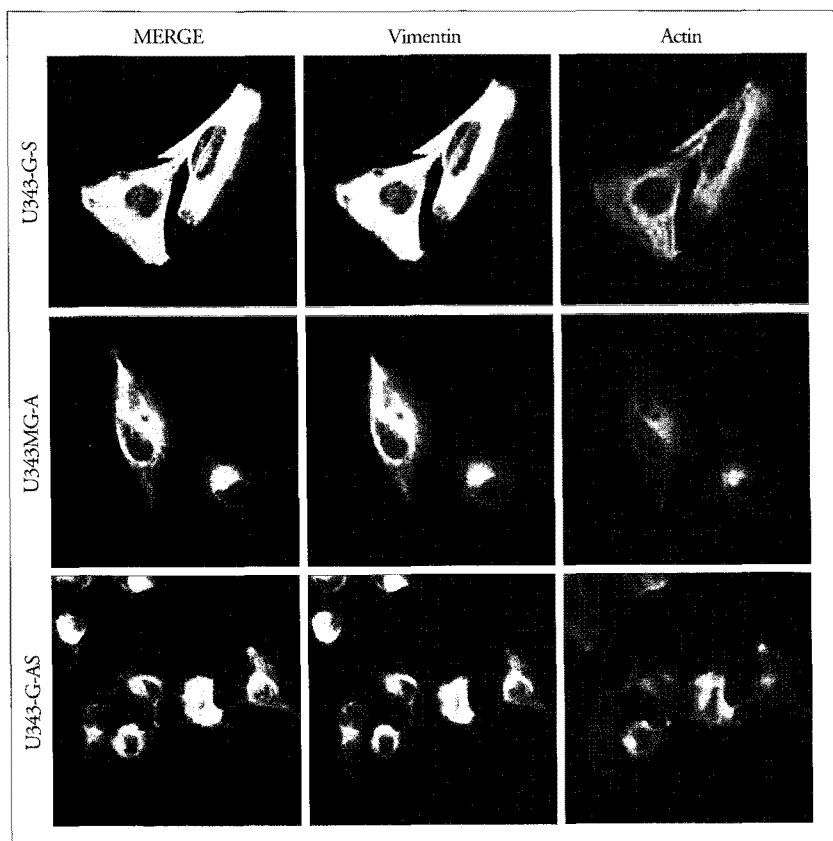


Fig. 5. Immunofluorescence staining shows the cytoskeletal changes after transfection (actin : red; vimentin : green).

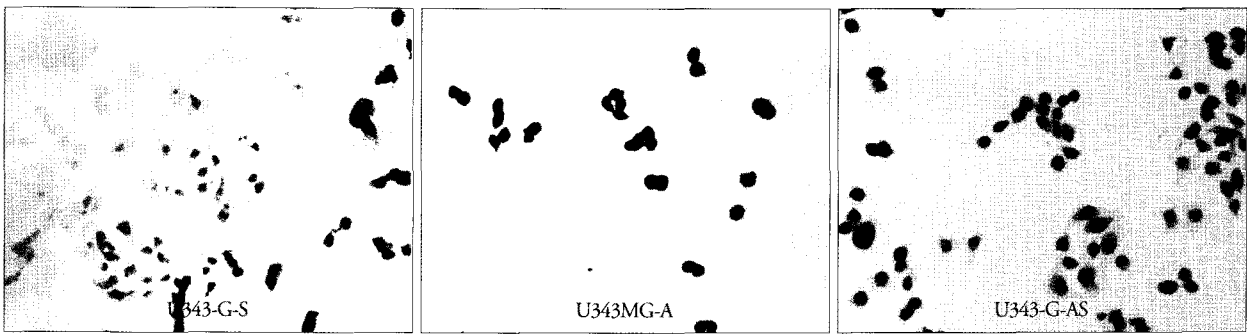


Fig. 6. The morphology of U343-G-S is smaller and rounded compared with U343MG-A (Fig. 5 and 6; staining with 0.1% toluidine blue; original magnification $\times 200$).

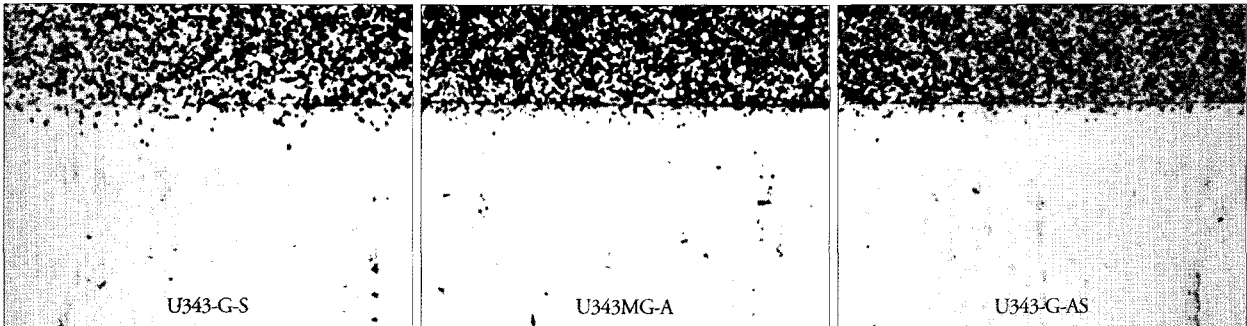


Fig. 7. Cell-migration tests by simple scratch technique show that the U343-G-S has more motility in terms of the distance of migration compared with U343MG-A and U343-G-AS, but there was no significant difference in the cell number ($p > 0.05$; staining with toluidine blue; original magnification $\times 40$).

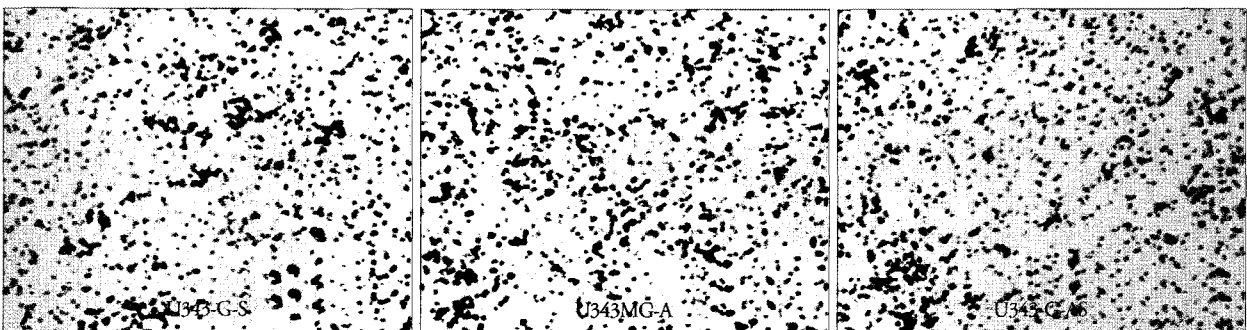


Fig. 8. In the Matrigel invasion assay, the mean number of invading cells was 110.3 ± 12.4 , 122.5 ± 16.1 , and 117.3 ± 14.6 for U343-G-S, U343MG-A, and U343-G-AS, respectively. There was no significant difference between the three cells ($p > 0.05$; staining with hemacolor; original magnification $\times 40$).

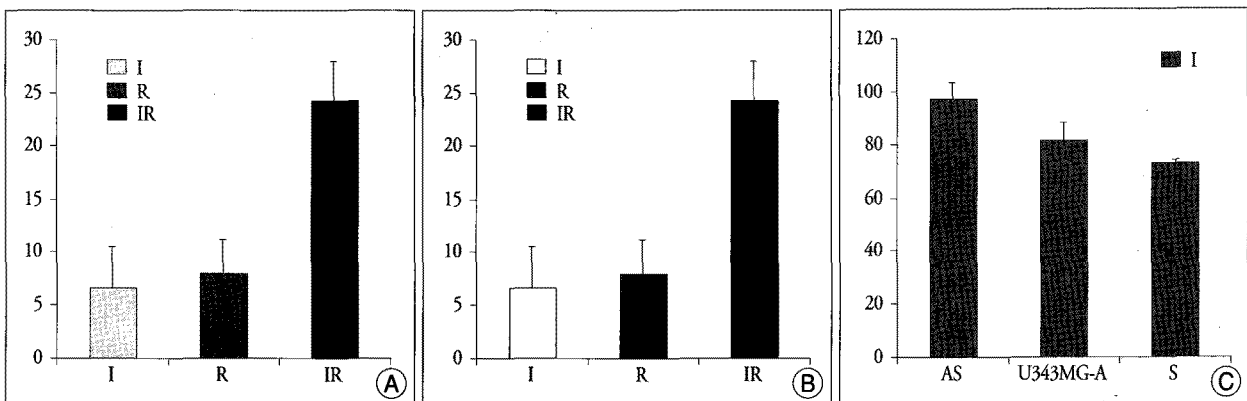


Fig. 9. MTT assay was performed to determine whether or not GRIM-19 is associated with the combination of INF and RA in the regulation of cell survival. A : U343-G-S cells treated for 24 hours with various concentrations of INF/RA. B : U343-G-S cells treated for 24 hours with INF/RA, INF, and RA. (I : INF 1,500 U only, R : RA 2 μ M only, IR : combination of INF 1,500 U and RA 2 μ M). C : U343-G-S, U343MG-A, and U343-G-AS cells treated for 24 hours with INF 1,500 U/RA 2 μ M. AS : U343-G-AS, S : U343-G-S.

in the G2/M phase (Fig. 10B). To identify the time of apoptosis, U343-G-S and U343-G-AS cells were treated with INF/RA, and a FACS analysis was performed at different time points. The untreated cells were designated as the control (Table 1). After 4 hours, the U343-G-S cells treated with INF- β /RA clearly showed early apoptosis (12.7%); after 12 hours, late apoptosis was significantly increased (7.48%), and after 24 hours, early apoptosis was significantly decreased (2.68%). At these different time points (4, 6, and 12 hours), the INF- β /RA-treated U343-G-S cells showed more apoptosis than the cells without treatment ($p = 0.01$). In contrast, the U343-G-AS cells showed no significant difference between the cells treated with INF- β /RA and those without treatment ($p > 0.05$).

DISCUSSION

GeneFishing™ Technology is a new and unique approach that is capable of identifying only authentic, differentially expressed genes (DEGs) in two or more nucleic acid samples. This technology dramatically improved the specificity of PCR amplification, enabling researchers to find authentic PCR products, and is known as a comprehensive screening method for tumor-related genes²³.

GRIM-19 encodes a 16-kDa protein consisting of 144 amino acids, and has been mapped to human chromosome 19p13.2⁶. GRIM-19 is expressed ubiquitously in various human tissues at a high level in the heart and skeletal muscle, and to a lesser extent in the liver, kidney, and brain. GRIM-19 is one of the GRIM¹⁶. GRIM-19 is a cell death regulatory gene that promotes apoptosis and a negative regulator of cell growth, and it is also involved in mitochondrial metabolism^{2,32}. It is the human homologue of the bovine subunit of mitochondrial nicotinamide adenine dinucleotide (NAD), i.e., ubiquinone oxidoreductase complex (complex I) of the mitochondrial respiratory chain (MRC)¹⁰.

Apoptosis is a physiologic process that regulates tissue homeostasis and inhibits neoplastic cell proliferation and

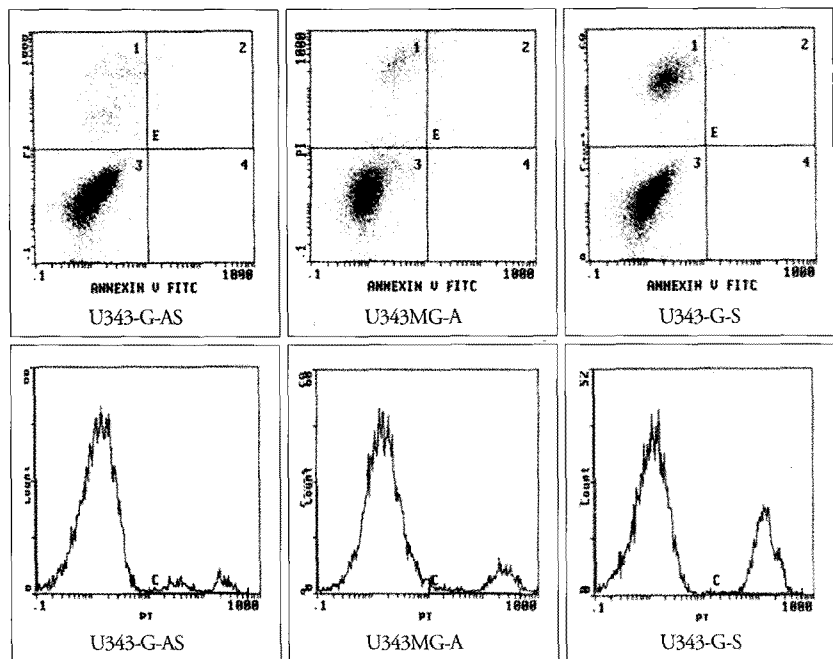


Fig. 10. FACS analysis shows the anti-proliferative activity of U343-G-S, U343MG-A, and U343-G-AS cells treated for 24 hours with INF/RA.

Table 1. U343-G-S and U343-G-AS cells were treated with the INF/RA combination and a FACS analysis was performed at different time points. The untreated cells were designated as the control

Cells	INF/RA		2 h	4 h	6 h	12 h	24 h	
U343-G-S	Untreated	Early	1.6	4.4	3.9	5.1	3.0	$p = 0.01$
		Late	2.7	2.0	2.7	3.7	2.4	
		Total	4.3	6.4	6.5	8.8	5.4	
	Treated	Early	4.2	12.7	10.1	10.1	2.7	
		Late	1.0	1.0	1.8	7.5	7.6	
		Total	5.2	13.6	11.8	17.6	10.3	
U343-G-AS	Untreated	Early	6.3	1.9	1.9	2.9	NA	$p > 0.05$
		Late	2.2	4.0	3.8	7.0	NA	
		Total	8.5	5.8	5.6	9.8	NA	
	Treated	Early	6.5	1.5	1.4	3.7	NA	
		Late	1.6	5.0	5.3	8.4	NA	
		Total	8.1	6.5	6.7	12.0	NA	

NA : not available, U343-G-S : sense GRIM-19 transfected U343MG-A, U343-G-AS : antisense GRIM-19 transfected U343MG-A

autoimmune disorders²⁵). A number of biologic response modifiers, such as cytokines and hormones, induce apoptosis in a cell type-specific manner. Although the core components of mammalian cell death pathways (Bcl proteins and caspases),^{3,25,33} are well-understood several recent studies have shown that apoptosis-like death occurs in a caspase or Bcl protein-independent manner²⁸. Thus, there could be several uncharacterized novel cell death regulators in mammals.

Interferons (IFNs) represent a family of proteins that regulate antiviral, antitumor, and immune responses in vertebrates^{21,41}. Type I (IFN- α and IFN- β) and type II (IFN- γ), the two major classes of IFNs, regulate these responses by inducing the expression of a number of cellular IFN-stimul-

ated genes upon binding to the specific high affinity cell surface receptors⁴⁰. Ligand-induced oligomerization causes tyrosine phosphorylation of IFN receptors by protein tyrosine kinases of the Janus tyrosine kinases (JAK) family. Subsequently, the signal transducing activators of transcription (STAT) proteins are phosphorylated by JAKs, causing migration of the activated STATs to the nucleus and the induction of gene expression. Several studies have shown that JAK1 and STAT1 are critical for type I and type II IFN-induced gene expression^{7,40}. Consistent with the importance of IFNs in cell growth control, the disruption of the STAT1 gene results in the loss of specific caspase genes of the apoptotic machinery²⁶. STAT1 is also critical for immune surveillance of neoplastic cells²². Because STAT1 is a transcription factor, the genes regulated by STAT1 ultimately mediate the growth-suppressive actions of IFN. A number of IFN-stimulated genes are thought to mediate the pleiotropic effects of various IFNs^{8,41}. The biological functions of many of these genes remain to be clarified.

RA, a metabolite of vitamin A, has a profound influence on cell growth, differentiation, and metabolism³¹. Prolonged deprivation of vitamin A in laboratory animals results in an increased incidence of spontaneous tumors, such as carcinomas. RA inhibits the growth of certain neuroblastomas, promyelocytic leukemias, and teratocarcinomas *in vitro*³¹. Clinically, retinoids have been shown to be effective in the therapy or prevention of primary cancers of the skin, head, and neck¹⁷. RA binds to specific nuclear retinoic acid receptors (RAR) and activates transcription³⁴. RAR acts as a transcription factor in association with a structurally similar, but genetically distinct dimerizing partner, the retinoid X receptor, by binding to a direct repeat element, the retinoic acid response element. Although the receptor complex is constitutively bound to the RA response element, several co-repressors that associate with this inhibit the gene expression complex in the unstimulated state¹². Upon engagement with a ligand, the co-repressors are dislodged from this complex, and this is followed by the recruitment of several co-activators that induce gene transcription¹². Genes controlled by this pathway are thought to mediate pleiotropic biological responses, including growth suppression³¹. In mammals, several isotypes of RARs and retinoid X receptors exist. The expression and function of RARs and retinoid X receptors are regulated in a tissue- and gene-specific manner³⁴. Several studies have shown the importance of these receptors in cell growth regulation^{9,34}. Like IFNs, retinoids induce the expression of a variety of genes^{31,34}. However, it is not clear which gene products inhibit tumor cell proliferation and apoptosis in response to retinoids.

Promyelocytic leukemias (PML)-RAR, a mutant retinoic

acid receptor found in certain acute promyelocytic leukemias, is generated by gene translocation^{29,46}. Because this receptor responds to RA, it is used as a target for therapy of acute promyelocytic leukemia with RA. Interestingly, this mutant receptor is induced by IFNs and has been reported to participate in the anti-cellular actions of IFN- α ^{4,5,38}. The mutant receptor forms a nuclear body consisting of several IFN-inducible gene products¹³. We and others have reported that in IFN-resistant cells, RA induces STAT1 levels, leading to an enhanced IFN response^{11,24,35}. These data suggest the existence of cross-talk between the IFN- and RA-stimulated pathways, although these ligands exert their effects via disparate signaling mechanisms.

The combination of IFN and retinoids is a more potent suppressor of cell growth *in vitro* and *in vivo* compared with either agent alone^{30,37}. To understand the mechanism underlying this synergistic effect, an antisense knockout approach was utilized to identify those genes that are involved in IFN- β -RA-inducible cell death in HeLa cells. These genes were identified and named GRIM¹⁶. GRIM-19 is one of these genes²; however, it is not clear how GRIM-19 promotes apoptosis.

Angell et al.² reported the identification of GRIM-19, a novel cell death-regulatory gene induced by the combination of interferon-beta and retinoic acid, using a genetic approach. They reported that IFN-/RA treatment increases the mRNA level of GRIM-19 in human breast carcinoma cell lines. Lufei et al.³² and Zhang et al.⁴⁸ demonstrated that the major role of GRIM-19 in the control of cell growth is exerted through STAT3, a transcription factor known to be inhibited by GRIM-19 binding. In subsequent studies, Hu et al.¹⁸ reported that some oncogenic proteins, such as the vIRF1 of human herpes virus-8 and the E6 oncogenes of high-risk human papilloma viruses, bind to GRIM-19 and inactivate it. Similarly, GW112, a protein found in human esophageal cancers, has been shown to inhibit the death activating function of GRIM-19⁴⁹. GRIM-19 associates with the serine protease, HtrA2, for the purpose of promoting cell death³³. Recently, mutations in the human GRIM-19 coding region have been found in Hürthle cell thyroid carcinomas³⁶. More recently, a proteomic analysis revealed the loss of GRIM-19 expression in human renal cell carcinomas¹.

In this study, the expression of GRIM-19 in the astrocytic tumor groups was higher than the normal groups. In the astrocytic tumor groups, the expression of GRIM-19 was higher with increasing tumor grade, based on RT-PCR. Using the MTT assay, the combination of INF and RA enhanced the anti-proliferative activity compared with using INF or RA separately. The U343-G-S cells were more sensitive to the combination of IFN and RA in terms of the anti-proliferative

activity. Our results suggest that GRIM-19 is associated with the anti-proliferative activity enhanced by the combination of INF and RA. Using FACS analysis, we observed that the cell survival rates are similar to the MTT assay and the anti-proliferative activity was caused by apoptosis when treated with the INF/RA combination. These suggest that GRIM-19 associated with the INF/RA combination could regulate cell death by inducing apoptosis in a glioblastoma cell line.

CONCLUSION

In astrocytic tumors, the expression of GRIM-19 was higher than normal brain tissue. The expression of GRIM-19 was also higher in high-grade tumors, and glioblastomas showed the highest expression. GRIM-19 associated with the INF/RA combination regulated cell death via apoptosis in human glioma cell lines. Therefore, GRIM-19 might be regarded as a tumor suppressor and expected to play a role in the future treatment of gliomas.

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