

IFN- β sensitizes TRAIL-induced apoptosis by upregulation of death receptor 5 in malignant glioma cells

SODAI YOSHIMURA¹, EMIKO SANNO², YUYA HANASHIMA¹, SHUN YAMAMURO¹, KOICHIRO SUMI¹, TAKUYA UEDA², TOMOHIRO NAKAYAMA³, HIROYUKI HARA⁴, ATSUO YOSHINO¹ and YOICHI KATAYAMA^{1,5}

¹Division of Neurosurgery, Department of Neurological Surgery, Nihon University School of Medicine, Tokyo 173-8610; ²Department of Computational Biology and Medical Science, Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa, Chiba 277-8562; ³Division of Companion Diagnostics, Department of Pathology and Microbiology, Nihon University School of Medicine; ⁴Division of Functional Morphology, Department of Functional Morphology, Nihon University School of Medicine, Tokyo 173-8610; ⁵Center for Brain and Health Science, Aomori University, Aomori 030-8505, Japan

Received April 24, 2019; Accepted September 30, 2019

DOI: 10.3892/or.2019.7383

Abstract. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor (TNF) family, induces apoptosis in cancer cells by binding to its receptors, death receptor 4 (DR4) and DR5, without affecting normal cells, and is therefore considered to be a promising antitumor agent for use in cancer treatment. However, several studies have indicated that most glioma cell lines display resistance to TRAIL-induced apoptosis. To overcome such resistance and to improve the efficacy of TRAIL-based therapies, identification of ideal agents for combinational treatment is important for achieving rational clinical treatment in glioblastoma patients. The main aim of this study was to investigate whether interferon- β (IFN- β) (with its pleiotropic antitumor activities) could sensitize malignant glioma cells to TRAIL-induced apoptosis using glioma cell lines. TRAIL exhibited a dose-dependent antitumor effect in all of the 7 types of malignant glioma cell lines, although the intensity of the effect varied among the cell lines. In addition, combined treatment with TRAIL (low clinical dose: 1 ng/ml) and IFN- β (clinically relevant concentration: 10 IU/ml) in A-172, AM-38, T98G, U-138MG and U-251MG demonstrated a more marked antitumor effect than TRAIL alone. Furthermore, the antitumor effect of the combined treatment with TRAIL and IFN- β may be enhanced via an extrinsic apoptotic system, and upregulation of DR5 was revealed to play an important role in this process in U-138MG cells. These findings provide an experimental basis to suggest that combined treatment with

TRAIL and IFN- β may offer a new therapeutic strategy for malignant gliomas.

Introduction

Malignant gliomas, especially glioblastomas, are the most frequent and devastating primary tumors of the central nervous system (1,2). Despite improvements in multimodality treatments consisting of combinations of surgical resection, irradiation, and chemotherapy, the prognosis for glioblastoma patients remains dismal (3,4). Novel treatment strategies for patients with glioblastomas are thus urgently required.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor (TNF) family, and induces apoptosis in cancer cells by binding to its receptors, death receptor 4 (DR4) and DR5 (5-9). Although DR4 and DR5 are highly expressed in cancer cells, such expression is limited in normal cells (10,11). TRAIL has thus been expected to represent one of the most promising agents for use in cancer treatment (5-8). In a study employing malignant glioma specimens, it was demonstrated that the expression of DR4 was 75% and that of DR5 was 95% (12). However, several investigations have indicated that most glioma cell lines display resistance to TRAIL-induced apoptosis (12,13). Recently, various combination treatments with other drugs as a sensitizer have been attempted and have yielded promising results (14-18). To overcome the resistance to TRAIL-induced apoptosis and to improve the efficacy of TRAIL-based therapies, identification of ideal agents for combination treatment is important for achieving rational clinical treatment in glioblastoma patients.

Human interferon- β (IFN- β) is a cytokine which belongs to the type I IFNs (19). It was originally identified as an antiviral agent, and has been revealed to exhibit pleiotropic antitumor activities including an anti-angiogenic activity, immunomodulatory activity, growth inhibition, and apoptosis induction (20,21). In addition to such multiple functions of IFN- β against human neoplasias, it can act as a drug

Correspondence to: Professor Atsuo Yoshino, Division of Neurosurgery, Department of Neurological Surgery, Nihon University School of Medicine, 30-1 Oyaguchi-Kamicho, Itabashi-ku, Tokyo 173-8610, Japan
E-mail: yoshino.atsuo@nihon-u.ac.jp

Key words: glioblastoma, TRAIL, interferon- β , apoptosis, DR5

sensitizer enhancing the antitumor effect when administered in combination with other agents in the treatment of malignant gliomas (22-26). IFN- β binds to the cell membrane receptor, interferon- α/β receptor (IFNAR), and acts by enhancing the expression of IFN-stimulated genes (ISGs) through the signal induction of the Janus kinase (JAK)/signal transducer and activator of the transcription (STAT) signaling pathway. To date, over 300 ISGs have been identified, including genes such as TRAIL and Fas, suggesting a strong involvement with exogenous apoptosis. Furthermore, several recent studies have demonstrated that the apoptotic activity of IFN depends partly on the TRAIL signaling pathway (27,28).

The aforementioned findings suggest that favorable therapeutic interactions could occur between TRAIL and IFN- β . Therefore, in the present study, the potential sensitizing effects of IFN- β towards TRAIL-induced apoptosis was investigated in malignant glioma cells aiming to provide an experimental basis for rational clinical treatments in glioblastoma patients.

Materials and methods

Cell lines, culture conditions and materials. Human malignant glioma cell lines A-172 (cell no. JCRB0228; lot no. 021999), AM-38 (cell no. IFO50492; lot no. 12082003), T98G (cell no. IFO50303; lot no. 1007), U-251MG (cell no. IFO50288; lot no. 12132002), and YH-13 (cell no. IFO50493; lot no. 1164) were purchased from Health Science Research Resources Bank (Sennan, Osaka, Japan). U-87MG (glioblastoma of unknown origin; cat. no. HTB-14; lot no. 2497162) and U-138MG (cat. no. HTB-16; lot no. 1104428) were purchased from the American Type Culture Collection (Manassas, VA, USA). In a previous study, we confirmed that *O*⁶-methylguanine-DNA methyltransferase (MGMT, a key factor of alkylating agents) is expressed in T98G, U-138MG and YH-13 cells by real-time RT-PCR and western blot analysis (29). Consistent with an earlier study (30), it was also confirmed that T98G (237 Met→Ile) and U-251MG (273 Arg→His) have a point mutation in the *p53* gene (data not shown).

Cells were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM; Nissui Pharmaceutical Co., Ltd.) supplemented with 10% fetal calf serum (FCS; Life Technologies; Thermo Fisher Scientific, Inc.) using plastic culture flasks (Corning, Inc.) in a 37°C-humidified incubator with 5% CO₂. Natural-type IFN- β (Toray Industries, Inc.) and TRAIL (Wako Pure Chemical Industries, Ltd.) were employed for the experiments.

Cell viability analysis. Cells were seeded at 1x10⁴ cells/well in 24-well plates. After 24 h of attachment, the cells were further incubated with fresh medium containing TRAIL, and/or IFN- β for 72 h. To determine the cell viability, the surviving cells in each well were counted using a Coulter Counter (Coulter Counter Z1; Beckman Coulter, Inc.) after confirming the presence of living cells with 0.45% trypan blue solution (Sigma-Aldrich; Merck KGaA). The experiments were repeated 6 times at each concentration.

Further treatment conditions were set with TRAIL at 1 ng/ml and IFN- β at 10 IU/ml, since the cell growth inhibitory effect was significant when TRAIL was at 1 ng/ml or more, and 10 IU/ml of IFN- β represents a clinically relevant concentration (26,31). In phase II RCS for non-small cell carcinoma

and B cell lymphoma, 8 mg/kg of TRAIL was administered, and its blood concentration reached about 80 μ g/ml (32,33). The dose of TRAIL (1 ng/ml) employed in the following experiments was thus considered to be a low clinical dose.

Since U-138MG displayed a marked antitumor effect at a small amount (0.1 and 1 ng/ml) of TRAIL when used in combination with 10 IU/ml of IFN- β , these cells were employed in the following experiments.

Analysis of apoptosis by flow cytometry. Cells were seeded at 1x10⁶ cells/well in 6-well plates (Corning, Inc.) and cultured for 24 h. Subsequently, the cells were further incubated with fresh medium (control), medium containing TRAIL (1 ng/ml) and/or IFN- β (10 IU/ml) for 72 h. The cells were washed with phosphate-buffered saline (PBS) and collected using trypsin-EDTA solution. After suspension with 100 μ l binding buffer, 5 μ l of Annexin V Alexa Fluor 488 conjugate (Invitrogen; Thermo Fisher Scientific, Inc.) and 10 μ l of propidium iodide solution (PI; Miltenyi Biotec, Inc.) were added, and the cells were incubated at room temperature for 15 min. Stained cells were analyzed with a fluorescence-activated cell sorter (FACS)-Calibur flow cytometer (BD Biosciences). The experiments were repeated 3 times to confirm reproducibility.

Western blot analysis. Proteins were isolated from 1x10⁷ cells using RIPA buffer (Wako Pure Chemical Industries, Ltd.) supplemented with protease inhibitor complex mix (Roche Diagnostics). The protein concentrations were determined using a Pierce BCA protein assay kit (Thermo Fisher Scientific, Inc.). A total of 50 μ g of protein was separated by 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (TEFCO, Inc.) and transferred onto nitrocellulose membranes (GE Healthcare) for 30 min at 15 V employing Bio-Rad Trans Blot (Bio-Rad Laboratories, Inc.). The membranes were blocked with 1% skimmed milk dissolved in washing buffer (PBS + 0.1% Tween-20) for 60 min at room temperature. The membranes were incubated with primary antibodies diluted according to the manufacturer's instructions at 4°C overnight (anti-caspase-3 rabbit mAb, cat. no. 9665; 1:1,000 dilution; anti-caspase-8 mouse mAb cat. no. 9746; 1:1,000 dilution; and anti-caspase-9 mouse mAb, cat. no. 9508; 1:1,000 dilution) (Cell Signaling Technology, Inc.). Anti- β -actin mouse mAb (cat. no. 013-24553; 1:2,000 dilution; Wako Pure Chemical Industries, Ltd.) was utilized as a loading control. Anti-mouse or anti-rabbit IgG (cat. no. A4416; 1:5,000 dilution; Sigma-Aldrich; Merck KGaA, cat. no. 7074; 1:5,000 dilution; Cell Signaling Technology, Inc., respectively) was employed as the secondary antibody for 60 min at room temperature. The band patterns were analyzed using ImageQuant LAS-4000 after treatment with ECL Prime Western Blotting Detection Reagent (both from GE Healthcare). The same experiments were repeated 3 times to confirm reproducibility.

Real-time reverse transcription-PCR analysis. RNA extraction was performed using an RNeasy Mini kit (Qiagen, Inc.). Relative mRNA expression was evaluated by real-time reverse transcription-PCR (qRT-PCR) employing a StepOne Real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) and a One-Step qPCR kit (SYBR[®] Green Real-time

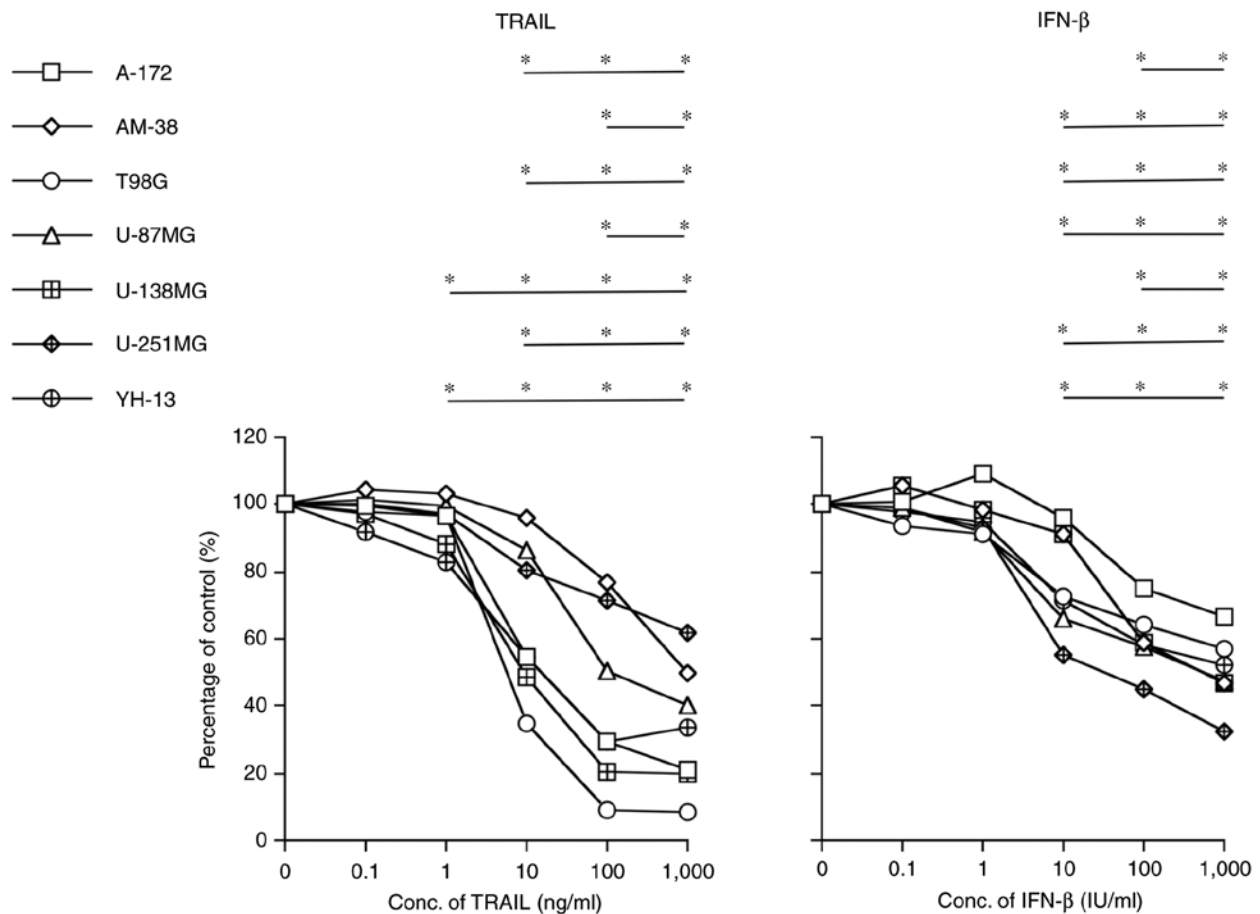


Figure 1. Antitumor effects of TRAIL (0-1,000 ng/ml) or IFN- β (0-1,000 IU/ml) against 7 types of malignant glioma cell lines (A-172, AM-38, T98G, U-87MG, U-138MG, U-251MG and YH-13). Both TRAIL (left) and IFN- β (right) exhibited a cell growth inhibitory effect in a dose-dependent manner in all cell lines at 72 h. Data are expressed as a percentage of the control. Compared to the no treatment group and the treatment groups, * $P < 0.05$. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; IFN- β , interferon- β .

PCR Master Mix (Toyobo Life Science) according to the manufacturers' instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was utilized as an internal control.

The following primers were used in this experiment (17,34-36): GAPDH forward, 5'-CAGAACATCATCCCTGCC TCT-3' and reverse, 5'-GCTTGACAAAGTGGTTCGTTGAG-3'; DR4 forward, 5'-TGTACGCCTGGAGTGACAT-3' and reverse, 5'-CACCAACAGCAACGGAACAA-3'; DR5 forward, 5'-CAG GTGTCAACATGTTGTCC-3' and reverse, 5'-ATCGAA GCATGTCTGAGAG-3'; cellular FLICE inhibitory protein (c-FLIP) forward, 5'-CGGACTATAGAGTGCTGATGG-3' and reverse, 5'-GATTATCAGGCAGATTCCCTAG-3'; p53 forward, 5'-GGCCCACTTCACCGTACTAA-3' and reverse, 5'-CTG GTTCAAGGCCAGAT GT-3'; and B-cell lymphoma 2-associated x protein (Bax) forward, 5'-TTTGCTTCAGGGTTTCAT CC-3' and reverse, 5'-CAGTTGAAGTTGCCGTCAGA-3'. The thermocycling conditions were 90°C for 30 sec, 61°C for 20 min, and 95°C for 1 min, followed by 40 cycles at 95°C for 15 sec, 55°C for 15 sec and 74°C for 45 sec. The expression levels were calculated employing the following equations by comparing the threshold cycle (CT): $\Delta Cq = CT$ of DR4, DR5, FAS, p53, Bax, or c-FLIP-CT of GAPDH, $\Delta\Delta Cq$ (target cell line)- ΔCq (reference cell line), and ratio = $2^{-\Delta\Delta Cq}$ (37). The experiments were repeated 3 times under each condition.

Influence of DR5 blocking antibody on the antitumor effect of combined treatment with TRAIL and IFN- β . In order to evaluate the involvement of DR5 in the antitumor effect of TRAIL in combination with IFN- β , the anti-apoptotic effect of administering DR5 blocking antibody was examined. U-138MG cells were seeded at 5×10^4 cells/well in 24-well plates. After 24 h of attachment, the cells were further incubated with fresh medium containing 10 ng/ml DR5 blocking antibody (Recombinant Human TRAIL R2/TNFRSF10B Fc Chimera Protein; R&D Systems, Inc.), or 1 ng/ml TRAIL and 10 IU/ml IFN- β without/with DR5 blocking antibody (2.5, 5 and 10 ng/ml) for 72 h. The surviving cells in each well were then counted using a Coulter Counter (Coulter Counter Z1; Beckman Coulter, Inc.) after confirming the presence of living cells with 0.45% trypan blue solution (Sigma-Aldrich; Merck KGaA). The experiments were repeated 6 times at each concentration.

Statistical analysis. The experiments were repeated at least 3 times under each condition. Where the same experiment was performed more than 6 times, the mean value and standard error (SE) were calculated and utilized. Mann-Whitney's U test was carried out to compare data between pairs of groups. The Tukey-Kramer test was conducted to perform comparisons of three or more groups, and when there was a significant

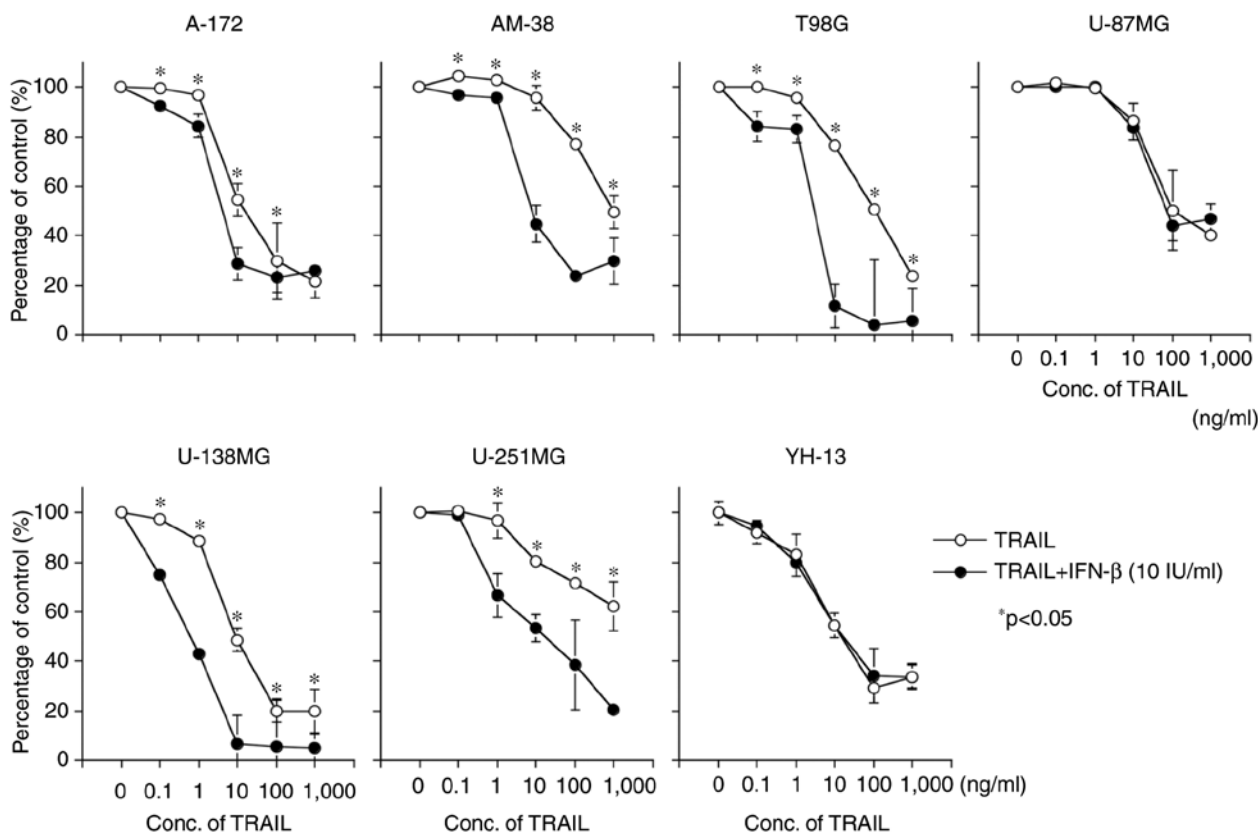


Figure 2. Cell growth inhibitory effect of a combination of TRAIL and IFN- β on malignant glioma cell lines. Seven malignant glioma cell lines (A-172, AM-38, T98G, U-87MG, U-138MG, U-251MG and YH-13) were exposed to medium containing various concentrations of TRAIL (0-1,000 ng/ml) without IFN- β (\circ) or with 10 IU/ml IFN- β (\bullet) for 72 h. The combined treatment with TRAIL and IFN- β revealed an additive cell growth inhibitory effect in A-172, AM-38, T98G, U-138MG, and U-251MG, whereas it did not do so in U-87MG and YH-13. Data are presented as the means \pm SE (standard error). *P<0.05. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; IFN- β , interferon- β .

difference, Student's t-test was employed as a subsequent test. For the data analyses, the statistical software SPSS (version 21.0; IBM Corp.) was used.

Results

Antitumor effect of TRAIL and IFN- β in malignant glioma cells. To evaluate the antitumor effect of TRAIL and IFN- β , the 7 malignant glioma cell lines (A-172, AM-38, T98G, U-87MG, U-138MG, U-251MG and YH-13) were treated with 0-1,000 ng/ml of TRAIL or 0-1,000 IU/ml of IFN- β for 72 h, and then assessed by counting the viable cells in the media. In all cell lines, both TRAIL and IFN- β caused a decrease in cell viability in a dose-dependent manner as revealed in Fig. 1.

Antitumor effect of a combination of TRAIL and IFN- β . To assess whether or not combined treatment with TRAIL and IFN- β could exert a synergistic effect in the 7 malignant glioma cell lines, cells were treated with various concentrations of TRAIL (0-1,000 ng/ml) or TRAIL with 10 IU/ml of IFN- β for 72 h. As revealed in Fig. 2, combined treatment with TRAIL and IFN- β revealed an additive cell growth inhibitory effect in the A-172, AM-38, T98G, U-138MG, and U-251MG cell lines, whereas no significant additive effect was observed in the U-87MG and YH-13 cell lines.

These results did not appear to be related to the MGMT or the *p53* status in the case of TRAIL or TRAIL and IFN- β

sensitivity. Because the additive cell growth inhibitory effect of combined treatment with TRAIL and IFN- β was observed with T98G and U-138MG, but not with YH-13, although these three cell lines are MGMT positive. In addition, the additive cell growth inhibitory effect of combined treatment with TRAIL and IFN- β was observed with the A-172, AM-38, T98G, U-138MG and U-251MG cell lines, although A-172, AM-38, U-87MG, U-138MG and YH-13 are wild-types of *p53* status, in contrast T98G and U-251MG are mutant types of *p53* status.

Detection of apoptosis by flow cytometry and western blotting. To assess the apoptosis induced by TRAIL and IFN- β , flow cytometry with Annexin V/PI double staining was performed (Annexin V-positive, early-stage apoptosis; Annexin V/PI-positive, late-stage apoptosis). As revealed in Fig. 3, combined treatment with TRAIL (1 ng/ml) and IFN- β (10 IU/ml) induced significant apoptosis after 72 h in U-138MG cells. The proportion of Annexin V/PI positive cells following combined treatment with TRAIL and IFN- β (mean = 7.99%) was higher than that for each single agent (means: Untreated, 2.71%; TRAIL, 3.77%; and IFN- β , 4.13%, respectively).

Furthermore, to evaluate the underlying mechanisms of the apoptotic effect of TRAIL and IFN- β , the protein expression of caspase-3 (an effector caspase), caspase-8 (extrinsic apoptotic pathway), and caspase-9 (intrinsic mitochondrial

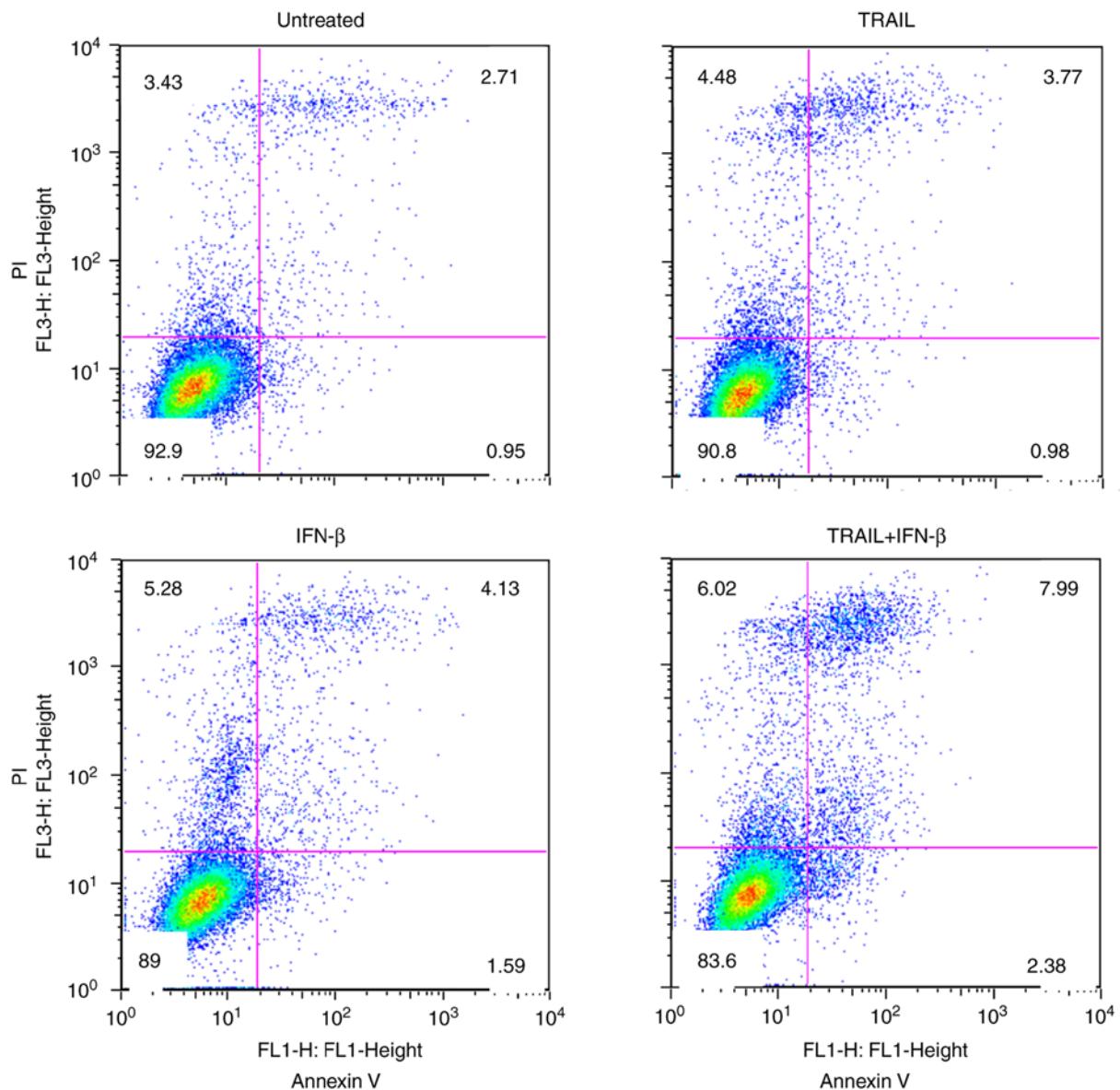


Figure 3. Induction of apoptosis by TRAIL (1 ng/ml) and/or IFN- β (10 IU/ml) in U-138MG cells as determined with FACS. The proportion of apoptotic cells (Annexin V-positive, early-stage apoptosis; Annexin V/PI-positive, late-stage apoptosis) was increased after 72 h of combined treatment with TRAIL and IFN- β in U-138MG cells. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; IFN- β , interferon- β .

pathway) were evaluated by western blot analysis. As revealed in Fig. 4, following 24 h of combined treatment with TRAIL and IFN- β , the protein expression levels of cleaved caspase-8 and cleaved caspase-3 were revealed to be more marked when compared to those following treatment with TRAIL or IFN- β alone. However, as regards the protein expression of caspase-9 and cleaved caspase-9, no difference was noted among the treatments. The effect of combined treatment with TRAIL and IFN- β in U-138MG may thus be due to promotion of apoptosis through the exogenous apoptotic pathway.

Expression of apoptosis-related genes. To further elucidate the role of IFN- β in combined treatment with TRAIL and IFN- β , the mRNA expression levels of apoptosis-related genes were evaluated by real-time qRT-PCR. The mRNA expression levels of *DR4*, *DR5*, *Fas*, *p53*, *Bax* and *c-FLIP* were determined following 4 h of IFN- β treatment in

U-138MG cells. Significant upregulation of *DR5* (mean: 2.97 ± 0.23 -fold), *Fas* (mean: 1.65 ± 0.11 -fold), and *p53* (mean: 1.50 ± 0.11 -fold) was detected; however, no significant changes in *DR4* (mean: 1.07 ± 0.01 -fold), *Bax* (mean: 1.17 ± 0.11 -fold), and *c-FLIP* (mean: 0.89 ± 0.23 -fold) were revealed (Fig. 5).

Apoptosis inhibition by DR5 blocking antibody. To confirm that the antitumor effect of combined treatment with TRAIL and IFN- β in malignant glioma cells is dependent on DR5, the number of surviving cells was counted after treatment for 72 h with DR5 blocking antibody (2.5, 5 or 10 ng/ml). The DR5 blocking antibody alone displayed no significant effect on U-138MG cells, and combined treatment with TRAIL (1 ng/ml) and IFN- β (10 IU/ml) exhibited a significant cell growth inhibitory effect (mean: $39.3 \pm 10.4\%$ when compared to no treatment). The DR5 blocking antibody caused a significant attenuation of the inhibitory effect on cell proliferation

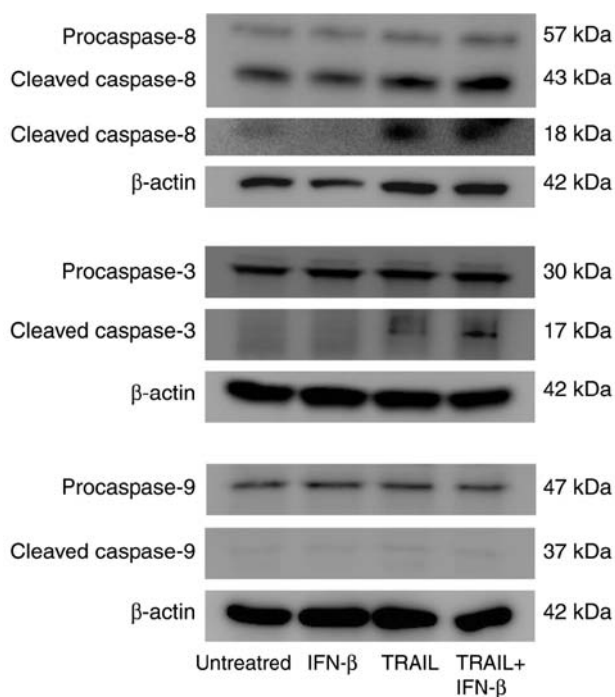


Figure 4. Apoptotic pathway induction by TRAIL and/or IFN- β . Western blot analysis revealed that the protein expression levels of cleaved caspase-8 and caspase-3 were more marked following combined treatment with TRAIL (1 ng/ml) and IFN- β (10 IU/ml) for 24 h as compared to treatment with TRAIL or IFN- β alone in U-138MG cells. However, concerning the protein expression of cleaved caspase-9, no difference was observed between the treatment groups. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; IFN- β , interferon- β .

in a concentration-dependent manner in U-138MG; the viable cells when 2.5, 5, or 10 ng/ml of DR5 blocking antibody was added to TRAIL and IFN- β combined treatment amounted to 54.8 \pm 9.0, 77.4 \pm 8.5 and 82.8 \pm 5.1%, respectively, as compared to the untreated case (Fig. 6).

Discussion

TRAIL has been considered to represent a promising agent for malignant glioma treatment, due to its ability to induce apoptosis of cancer cells without affecting normal cells (10,11). However, several studies have suggested that most glioma cells display resistance to TRAIL-induced apoptosis (12,13), and to overcome this resistance is a critical issue. Among various investigations conducted, it has been reported that TRAIL-induced apoptosis can be enhanced by combination with other drugs or by irradiation (14-18,38). Combined treatment with TRAIL sensitizers is regarded as a promising strategy to overcome TRAIL resistance, and several attempts have been made to identify such TRAIL sensitizers (14-18).

In the present study, TRAIL exhibited a dose-dependent antitumor effect in all of 7 types of malignant glioma cell lines, although the intensity of the effect varied among the cell lines. This finding is consistent with those obtained in previous studies and suggests that sensitivity/resistance to TRAIL differs among malignant gliomas (39-41). Furthermore, the combination treatment with TRAIL and IFN- β in 5 of the 7 malignant glioma cell lines demonstrated a more significant antitumor

effect than TRAIL alone. To the best of our knowledge, this is the first time that IFN- β enhancement of TRAIL-induced apoptosis has been observed in particular malignant glioma cells. In the future, one important issue requiring examination will be the antitumor effect occurring after application of TRAIL and IFN- β in combination with TMZ, which is the current standard therapeutic agent for malignant gliomas. In addition, once sufficient data have been gathered, the morphological changes of the cells associated with TRAIL, IFN- β , and combined treatment will require investigation.

Higuchi and Hashida (31) reported that the plasma concentration levels of IFN- β were 40 and 96 IU/ml following intravenous administration of 3 \times 10⁶ and 6 \times 10⁶ IU IFN- β , respectively, when administered in 60 min. On this basis, 10 IU/ml is considered a clinically relevant concentration of IFN- β . Furthermore, in phase II RCS for non-small cell lung cancer and B cell lymphoma, 8 mg/kg of TRAIL was administered, and its plasma concentration reached ~80 mg/ml (32,33). The concentration of TRAIL used in the present study is therefore considered to be clinically low. In particular, in A-172, AM-38, T98G, and U-138MG cells, a cell growth inhibitory effect was also significantly observed even at a small amount (0.1 and 1 ng/ml) of TRAIL with 10 IU/ml of IFN- β , suggesting that IFN- β represents a good candidate for use in combination with TRAIL-based treatment regimens for malignant gliomas. However, based on the present study, we do not have sufficient data to elucidate the exact differences in sensitivity to each drug between each cell line. Furthermore, the detailed mechanisms underlying such differences remain unidentified in literature, and this presents our next topic of research. In the present study, the focus was on evaluating in more detail how the combined treatment with TRAIL and IFN- β can provide a more marked antitumor effect.

Following combined treatment with TRAIL (1 ng/ml) and IFN- β (10 IU/ml), FACS analysis indicated that late-phase apoptosis or necrosis was increased (42), when compared to that with TRAIL or IFN- β alone in U-138MG cells. Furthermore, it was revealed by western blotting that caspase-8 and caspase-3, which are involved in the extrinsic apoptotic pathways (17,34-46), were cleaved by combined treatment with TRAIL and IFN- β , whereas caspase-9 which is involved in the intrinsic apoptotic pathway exhibited no cleavage. The antitumor effect of combined treatment with TRAIL and IFN- β may thus be enhanced via an extrinsic apoptotic system in U-138MG cells.

TRAIL induces apoptosis of cancer cells by binding to its receptors DR4 and DR5 (9). In malignant glioma cells, there have been indications that cisplatin and irradiation may upregulate the expression of DR5 (38,47). Conversely, in malignant glioma cells, IFN- β has been revealed to upregulate the expression of p53 (24). Furthermore, it has been suggested that p53 promotes the expression of DR5 (48). In the present study, it was demonstrated and confirmed that the quantitative p53 and DR5 mRNA levels were upregulated by IFN- β (10 IU/ml), however, no significant upregulation was observed for DR4 in U-138MG cells. In the future, it is surmised that it will also be necessary to investigate the protein levels produced by such apoptosis-related genes following IFN- β treatment. In addition, as revealed in Fig. 6, the antitumor effect elicited by the combined treatment with TRAIL and IFN- β was significantly

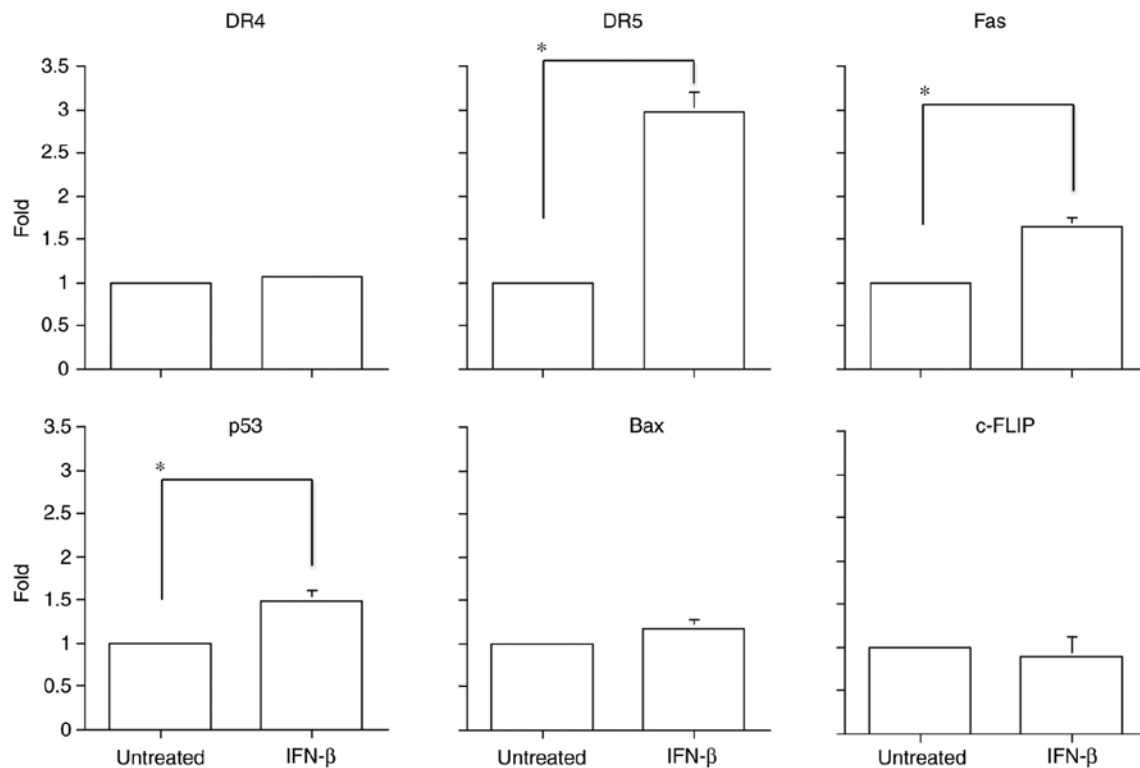


Figure 5. Apoptosis-related mRNA expression levels of *DR4*, *DR5*, *Fas*, *p53*, *Bax*, and *c-FLIP* following IFN- β treatment as analyzed by real-time qRT-PCR. Significant upregulation of *DR5*, *Fas*, and *p53* was observed in the case of IFN- β (10 IU/ml) treatment for 4 h in U-138MG cells. However, there were no differences in *DR4*, *Bax*, and *c-FLIP* following 4 h of IFN- β treatment in U-138MG cells. Data are presented as the means \pm SE (standard error). * $P < 0.05$ (Student's t-test). DR4, death receptor 4; DR5, death receptor 5; *c-FLIP*, cellular FLICE inhibitory protein; Bax, B-cell lymphoma 2-associated x protein; IFN- β , interferon- β .

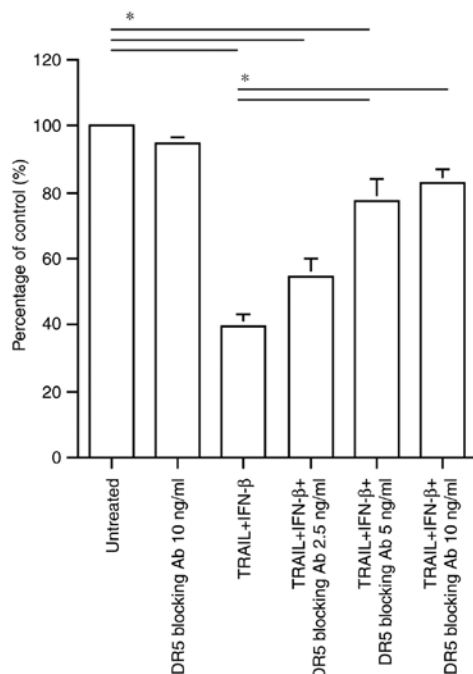


Figure 6. Cell growth after treatment with DR5 blocking antibody. The DR5 blocking antibody (10 ng/ml) alone displayed no significant effect on U-138MG cells after 72 h. The antibody significantly attenuated the antitumor effect of combined treatment with TRAIL (1 ng/ml) and IFN- β (10 IU/ml) in a dose-dependent manner after 72 h in U-138MG cells. Data are presented as the means \pm SE (standard error). The Tukey-Kramer test was used to identify which group differences were significant. * $P < 0.05$. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; IFN- β , interferon- β ; DR5, death receptor 5.

attenuated depending on the concentration of DR5 blocking antibody. These findings indicated that upregulation of DR5 via p53 by IFN- β may play an important role in the enhanced antitumor effect of combined treatment with TRAIL and IFN- β in U-138MG, although the investigation of the effect of DR5 blocking antibody with TRAIL or IFN- β treatment alone is required. Moreover, Fas, which is a receptor that induces apoptosis (extrinsic apoptotic pathway) (46), was also associated with increased expression levels of quantitative mRNA by IFN- β in the present study. These circumstances were considered to represent part of the mechanism whereby the antitumor effect was enhanced by employing IFN- β in combination with TRAIL.

Finally, *c-FLIP* is expressed in tumor cells to a certain level, and competes with caspase-8 to inhibit apoptosis by binding to Fas-associated death domain (49,50). It has been reported that various drugs lower the expression of *c-FLIP*, and the induction of apoptosis by TRAIL could be enhanced (49-53). It was therefore investigated whether the expression of *c-FLIP* was decreased by the administration of IFN- β . However, in the present study, no significant decrease in quantitative *c-FLIP* mRNA level by IFN- β was observed in U-138MG cells.

In conclusion, in the present study, it was demonstrated that combined treatment with a clinically relevant concentration of TRAIL and IFN- β produced a significantly enhanced antitumor effect in malignant glioma cells as compared to that achieved when either agent was used alone, and this may be partially dependent on DR5 through the extrinsic pathway of apoptosis. Although the present findings were not sufficient

to yield a conclusive theory of the mechanism of action, and *in vivo* experiments will also need to be undertaken in the future, the data did provide an experimental basis to suggest that combined treatment with TRAIL and IFN- β could offer a new therapeutic strategy for malignant gliomas.

Acknowledgements

The authors are grateful to Mr. Hiroyuki Satake and Mr. Nobuo Miyazaki, Toray Industries Inc. (Tokyo, Japan) for their invaluable discussions. Some parts of this study have been incorporated within a Japanese-language thesis submitted for Sodai Yoshimura's Ph.D. degree at Nihon University School of Medicine (Tokyo, Japan).

Funding

The present study was supported in part by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (grant no. 16K10772) and in part by a grant from the Health Sciences Research Institute, Inc. (Yokohama, Japan) for the Division of Companion Diagnostics, Department of Pathology and Microbiology, Nihon University School of Medicine (Tokyo, Japan).

Availability of data and materials

All data related to this study are included in this article.

Authors' contributions

SYo and ES developed the experimental design, performed most of the experiments and analysis, and drafted the basic manuscript. YH and SYa were involved in the conception and design of the study, undertook part of the experiments, analyzed the data, and contributed to the writing of the draft manuscript. KS also conducted some experiments and analyzed the data. TU, TN, HH, and YK were also involved in the conception and design of the study and proofread the manuscript. AY contributed to the experimental design and the writing of the manuscript. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests with regard to the subjects discussed in this study. TU received funds for other research projects not related to this study from the Ministry of Education, Culture, Sports, Science and Technology, Japan, the Ministry of Economy, Trade and

Industry, Japan and the Human Frontier Science Program, while AY received research funds for another research project from Medtronic Japan Co., Ltd.

References

- Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, Ohgaki H, Wiestler OD, Kleihues P and Ellison DW: The 2016 World Health Organization Classification of Tumors of the Central Nervous System: A summary. *Acta Neuropathol* 131: 803-820, 2016.
- Report of brain tumor registry of Japan (2015-2017). *Neuro Med Chir (Tokyo)* 59 (Suppl): S1-S81, 2019.
- Maher EA, Furnari FB, Bachoo RM, Rowitch DH, Louis DN, Cavenee WK and DePinho RA: Malignant glioma: Genetics and biology of a grave matter. *Genes Dev* 15: 1311-1333, 2001.
- Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, *et al*: Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* 352: 987-996, 2005.
- Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A and Ashkenazi A: Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J Biol Chem* 271: 12687-12690, 1996.
- Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, Chin W, Jones J, Woodward A, Le T, *et al*: Tumor necrosis factor-related apoptosis-inducing ligand *in vivo*. *Nat Med* 5: 157-163, 1999.
- Ashkenazi A, Holland P and Eckhardt SG: Ligand-based targeting of apoptosis in cancer: The potential of recombinant human apoptosis ligand 2/tumor necrosis factor-related apoptosis-inducing ligand (rhApo2L/TRAIL). *J Clin Oncol* 26: 3621-3630, 2008.
- Naoum GE, Buchsbaum DJ, Tawadros F, Farooqi A and Arafat WO: Journey of TRAIL from bench to bedside and its potential role in immuno-oncology. *Oncol Rev* 11: 332, 2017.
- von Karstedt S, Montinaro A and Walczak H: Exploring the TRAILs less travelled: TRAIL in cancer biology and therapy. *Nat Rev Cancer* 17: 352-366, 2017.
- Suliman A, Lam A, Datta R and Srivastava RK: Intracellular mechanisms of TRAIL: Apoptosis through mitochondrial-dependent and -independent pathways. *Oncogene* 20: 2122-2133, 2001.
- Nagane M, Huang HJ and Cavenee WK: The potential of TRAIL for cancer chemotherapy. *Apoptosis* 6: 191-197, 2001.
- Kuijlen JM, Bremer E, Mooij JJ, den Dunnen WF and Helfrich W: Review: On TRAIL for malignant glioma therapy? *Neuropathol Appl Neurobiol* 36: 168-182, 2010.
- Hawkins CJ: TRAIL and malignant glioma. *Vitam Horm* 67: 427-452, 2004.
- Shang Z and Zhang L: Digitoxin increases sensitivity of glioma stem cells to TRAIL-mediated apoptosis. *Neurosci Lett* 653: 19-24, 2017.
- Khan M, Bi Y, Qazi JI, Fan L and Gao H: Evodiamine sensitizes U87 glioblastoma cells to TRAIL via the death receptor pathway. *Mol Med Rep* 11: 257-262, 2015.
- Badr CE, Wurdinger T, Nilsson J, Niers JM, Whalen M, Degtrev A and Tannous BA: Lanatoside C sensitizes glioblastoma cells to tumor necrosis factor-related apoptosis-inducing ligand and induces an alternative cell death pathway. *Neuro Oncol* 13: 1213-1224, 2011.
- Yoon MJ, Kang YJ, Kim IY, Kim EH, Lee JA, Lim JH, Kwon TK and Choi KS: Monensin, a polyether ionophore antibiotic, overcomes TRAIL resistance in glioma cells via endoplasmic reticulum stress, DR5 upregulation and c-FLIP downregulation. *Carcinogenesis* 34: 1918-1928, 2013.
- Calzolari A, Saulle E, De Angelis ML, Pasquini L, Boe A, Pelacchi F, Ricci-Vitiani L, Baiocchi M and Testa U: Salinomycin potentiates the cytotoxic effects of TRAIL on glioblastoma cell lines. *PLoS One* 9: e94438, 2014.
- Borden EC, Sen GC, Uze G, Silverman RH, Ransohoff RM, Foster GR and Stark GR: Interferons at age 50: Past, current and future impact on biomedicine. *Nat Rev Drug Discov* 6: 975-990, 2007.
- Isaacs A and Lindenmann J: Virus interference. I. The interferon. *Proc R Soc Lond B Biol Sci* 147: 258-267, 1957.
- Vannucchi S, Chiantore MV, Mangino G, Percario ZA, Affabris E, Fiorucci G and Romeo G: Perspectives in biomolecular therapeutic intervention in cancer: From the early to the new strategies with type I interferons. *Curr Med Chem* 14: 667-679, 2007.

22. Yoshida J, Kajita Y, Wakabayashi T and Sugita K: Long-term follow-up results of 175 patients with malignant glioma: Importance of radical tumor resection and postoperative adjuvant therapy with interferon, ACNU and radiation. *Acta Neurochir (Wien)* 127: 55-59, 1994.
23. Watanabe T, Katayama Y, Yoshino A, Komine C, Yokoyama T and Fukushima T: Treatment of low-grade diffuse astrocytomas by surgery and human fibroblast interferon without radiation therapy. *J Neurooncol* 61: 171-176, 2003.
24. Natsume A, Ishii D, Wakabayashi T, Tsuno T, Hatano H, Mizuno M and Yoshida J: IFN-beta down-regulates the expression of DNA repair gene MGMT and sensitizes resistant glioma cells to temozolomide. *Cancer Res* 65: 7573-7579, 2005.
25. Watanabe T, Katayama Y, Yoshino A, Fukaya C and Yamamoto T: Human interferon beta, nimustine hydrochloride, and radiation therapy in the treatment of newly diagnosed malignant astrocytomas. *J Neurooncol* 72: 57-62, 2005.
26. Yoshino A, Ogino A, Yachi K, Ohta T, Fukushima T, Watanabe T, Katayama Y, Okamoto Y, Naruse N and Sano E: Effect of IFN-beta on human glioma cell lines with temozolomide resistance. *Int J Oncol* 35: 139-148, 2009.
27. Der SD, Zhou A, Williams BR and Silverman RH: Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. *Proc Natl Acad Sci USA* 95: 15623-15628, 1998.
28. Chawla-Sarkar M, Lindner DJ, Liu YF, Williams BR, Sen GC, Silverman RH and Borden EC: Apoptosis and interferons: Role of interferon-stimulated genes as mediators of apoptosis. *Apoptosis* 8: 237-249, 2003.
29. Yoshino A, Ogino A, Yachi K, Ohta T, Fukushima T, Watanabe T, Katayama Y, Okamoto Y, Naruse N, Sano E and Tsumoto K: Gene expression profiling predicts response to temozolomide in malignant gliomas. *Int J Oncol* 36: 1367-1377, 2010.
30. Wischhusen J, Naumann U, Ohgaki H, Rastinejad F and Weller M: CP-31398, a novel p53-stabilizing agent, induces p53-dependent and p53-independent glioma cell death. *Oncogene* 22: 8233-8245, 2003.
31. Higuchi Y and Hashida M: Pharmacokinetics of interferon. *Clinic All-Round* 52: 2499-2505, 2003 (In Japanese).
32. Soria JC, Márk Z, Zatloukal P, Szima B, Albert I, Juhász E, Pujol JL, Kozielski J, Baker N, Smethurst D, *et al*: Randomized phase II study of dulanermin in combination with paclitaxel, carboplatin, and bevacizumab in advanced non-small-cell lung cancer. *J Clin Oncol* 29: 4442-4451, 2011.
33. Cheah CY, Belada D, Fanale MA, Janikova A, Czucman MS, Flinn IW, Kapp AV, Ashkenazi A, Kelley S, Bray GL, *et al*: Dulanermin with rituximab in patients with relapsed indolent B-cell lymphoma: An open-label phase 1b/2 randomised study. *Lancet Haematol* 2: e166-e174, 2015.
34. Ray P, Guha D, Chakraborty J, Banerjee S, Adhikary A, Chakraborty S, Das T and Sa G: Crocetin exploits p53-induced death domain (PIDD) and FAS-associated death domain (FADD) proteins to induce apoptosis in colorectal cancer. *Sci Rep* 6: 32979, 2016.
35. Edagawa M, Kawauchi J, Hirata M, Goshima H, Inoue M, Okamoto T, Murakami A, Maehara Y and Kitajima S: Role of ATF3 for ER stress-induced sensitization of p53-deficient human colon cancer cells to TRAIL-mediated apoptosis through upregulation of DR5 by zerumbone and celecoxib. *J Biol Chem* 289: 21544-21561, 2014.
36. Earel JK Jr, VanOosten RL and Griffith TS: Histone deacetylase inhibitors modulate the sensitivity of tumor necrosis factor-related apoptosis-inducing ligand-resistant bladder tumor cells. *Cancer Res* 66: 499-507, 2006.
37. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
38. Liu J, Gao Q, Xie T, Liu Y, Luo L, Xu C, Shen L, Wan F, Lei T and Ye F: Synergistic effect of TRAIL and irradiation in elimination of glioblastoma stem-like cells. *Clin Exp Med* 18: 399-411, 2018.
39. Röhn TA, Wagenknecht B, Roth W, Naumann U, Gulbins E, Krammer PH, Walczak H and Weller M: CCNU-dependent potentiation of TRAIL/Apo2L-induced apoptosis in human glioma cells is p53-independent but may involve enhanced cytochrome c release. *Oncogene* 20: 4128-4137, 2001.
40. Hao C, Beguinot F, Condorelli G, Trencia A, Van Meir EG, Yong VW, Parney IF, Roa WH and Petruk KC: Induction and intracellular regulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) mediated apoptosis in human malignant glioma cells. *Cancer Res* 61: 1162-1170, 2001.
41. Xiao C, Yang BF, Asadi N, Beguinot F and Hao C: Tumor necrosis factor-related apoptosis-inducing ligand-induced death-inducing signaling complex and its modulation by c-FLIP and PED/PEA-15 in glioma cells. *J Biol Chem* 277: 25020-25025, 2002.
42. Rieger AM, Nelson KL, Konowalchuk JD and Barrada DR: Modified annexin V/propidium iodide apoptosis assay for accurate assessment of cell death. *J Vis Exp* 24, 2011.
43. Cohen GM: Caspases: The executioners of apoptosis. *Biochem J* 326: 1-16, 1997.
44. Green DR: Apoptotic pathways: Paper wraps stone blunts scissors. *Cell* 102: 1-4, 2000.
45. Fulda S and Debatin KM: Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene* 25: 4798-4811, 2006.
46. Elmore S: Apoptosis: A review of programmed cell death. *Toxicol Pathol* 35: 495-516, 2007.
47. Ding L, Yuan C, Wei F, Wang G, Zhang J, Bellail AC, Zhang Z, Olson JJ and Hao C: Cisplatin restores TRAIL apoptotic pathway in glioblastoma-derived stem cells through up-regulation of DR5 and down-regulation of c-FLIP. *Cancer Invest* 29: 511-520, 2011.
48. Wu GS, Burns TF, McDonald ER III, Jiang W, Meng R, Krantz ID, Kao G, Gan DD, Zhou JY, Muschel R, *et al*: KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. *Nat Genet* 17: 141-143, 1997.
49. Huang Y, Yang X, Xu T, Kong Q, Zhang Y, Shen Y, Wei Y, Wang G and Chang KJ: Overcoming resistance to TRAIL-induced apoptosis in solid tumor cells by simultaneously targeting death receptors, c-FLIP and IAPs. *Int J Oncol* 49: 153-163, 2016.
50. Min KJ, Um HJ, Seo SU, Woo SM, Kim S, Park JW, Lee HS, Kim SH, Choi YH, Lee TJ and Kwon TK: Angelicin potentiates TRAIL-induced apoptosis in renal carcinoma Caki cells through activation of caspase 3 and down-regulation of c-FLIP expression. *Drug Dev Res* 79: 3-10, 2018.
51. Lemke J, von Karstedt S, Abd El Hay M, Conti A, Arce F, Montinaro A, Papenfuss K, El-Bahrawy MA and Walczak H: Selective CDK9 inhibition overcomes TRAIL resistance by concomitant suppression of cFlip and Mcl-1. *Cell Death Differ* 21: 491-502, 2014.
52. Woo SM, Min KJ, Seo BR and Kwon TK: YM155 sensitizes TRAIL-induced apoptosis through cathepsin S-dependent down-regulation of Mcl-1 and NF-κB-mediated down-regulation of c-FLIP expression in human renal carcinoma Caki cells. *Oncotarget* 7: 61520-61532, 2016.
53. Irmiler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer JL, Schröter M, Burns K, Mattmann C, *et al*: Inhibition of death receptor signals by cellular FLIP. *Nature* 388: 190-195, 1997.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.