

Interferon- β sensitizes human malignant melanoma cells to temozolomide-induced apoptosis and autophagy

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Abstract. Malignant melanoma is a highly aggressive skin cancer that is highly resistant to chemotherapy. Adjuvant therapy is administered to patients with melanoma that possess no microscopic metastases or have a high risk of developing microscopic metastases. Methylating agents, including dacarbazine (DTIC) and temozolomide (TMZ), pegylated interferon (IFN)- α 2b and interleukin-2 have been approved for adjuvant immuno-chemotherapy; however, unsatisfactory results have been reported following the administration of methylating agents. IFN- β has been considered to be a signaling molecule with an important therapeutic potential in cancer. The aim of the present study was to elucidate whether antitumor effects could be augmented by the combination of TMZ and IFN- β in malignant melanoma. We evaluated the efficacy of TMZ and IFN- β by comparing O⁶-methylguanine-DNA transferase (MGMT)-proficient and -deficient cells, as MGMT has been reported to be associated with the resistance to methylating agents. Cell viability was determined by counting living cells with a Coulter counter, and apoptosis was analyzed by dual staining with Annexin V Alexa Fluor[®] 488 and propidium iodide. The expression of proteins involved in the cell cycle, apoptosis and autophagy was evaluated by western blot analysis. The combined treatment with TMZ and IFN- β suppressed cell proliferation and induced cell cycle arrest. We also demonstrated that a

combination of TMZ and IFN- β enhanced apoptosis and autophagy more efficiently compared with TMZ treatment alone. These findings suggest that antitumor activity may be potentiated by IFN- β in combination with TMZ.

Introduction

Malignant melanoma, which is derived from melanocytes, is a highly aggressive skin cancer. The incidence of malignant melanoma continues to increase worldwide (1). Postoperative adjuvant therapy is important in order to prevent tumor recurrence and metastasis (2). Various modalities, including immunological therapy, chemotherapy and radiation have been investigated as adjuvant therapies; however, the effects of advanced melanoma treatment remain unsatisfactory. Recent advances have demonstrated that immunotherapies using immune checkpoint inhibitors, including anti-cytotoxic T lymphocyte antigen-4 and anti-programmed death-1 antibodies, or molecularly-targeted agents, such as *BRAF* and *MEK* inhibitors, can markedly alter the effects of melanoma therapy. Of note, these agents exhibit only a transient benefit in the majority of cases (3-5) and patients suffer from the rapid onset of resistance (6). In addition, the financial cost is considerably high and government finances are restricted. Thus, there is an urgent need for novel agents or the novel application of current agents to overcome this aggressive malignancy (7).

Temozolomide (3-methyl-4-oxo-3,4-dihydro-imidazo [5,1-*d*] [1,2,3,5]tetrazine-8-carboxamide; TMZ), is an oral alkylating agent employed for the treatment of metastatic melanoma and malignant glioma (8). TMZ, as compared with dacarbazine (DTIC), is very well tolerated and possesses an advantage in terms of improving the quality of life of patients with metastatic melanoma (9). DTIC and TMZ are the most commonly used drugs as first-line therapy for patients with wild-type *BRAF* tumors in Europe (10). The effect of TMZ has been hypothesized to be dependent on the tumoral expression of O⁶-methylguanine-DNA transferase (MGMT),

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and low expression of MGMT has been correlated with a high likelihood of the increased sensitivity to TMZ (11,12). MGMT may be a key determinant of cellular resistance to methylating drugs; however, there is no significant association between MGMT expression and the therapeutic response in melanoma (13-15).

Interferons (IFNs) are cytokines; there are two groups of IFNs: type I (IFN- α , IFN- β , IFN- τ and IFN- ω) and type II (IFN- γ) (16). IFN- α 2b and pegylated IFN- α 2b have been approved in numerous countries as an adjuvant therapy for malignant melanoma (17). On the other hand, IFN- β is known to exhibit pleiotropic biological activities, including antiviral, antiproliferative, antiangiogenic and immunomodulatory effects (18). IFN- β also acts as a drug sensitizer to enhance toxicity against a variety of neoplasias when administered in combination with alkylating agents (19). IFN- β exhibits greater growth inhibitory and proapoptotic effects than IFN- α 2 (20).

TMZ in combination with IFN- α 2 has been investigated in metastatic melanomas (21-25). Treatment with a combination of TMZ and IFN- α can increase the median survival of patients with metastatic melanomas (26); however, a combination treatment with TMZ and IFN- β has rarely been investigated in malignant melanomas (15). In the present study, TMZ and IFN- β was applied to determine whether this combination can exert an antitumor activity on malignant melanomas.

Materials and methods

Materials. Natural-type of IFN- β (Toray Industries, Tokyo, Japan), TMZ (Tokyo Chemical Industry, Tokyo, Japan) and O⁶-benzylguanine (O⁶-BG; Abcam, Cambridge, UK) were used for the experiments.

Cell lines. Human malignant melanoma A375, CRL-1579, G361, MeWo and SK-MEL-28 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were routinely cultured in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA) using plastic culture flasks (Corning Inc., Corning, NY, USA) in a humidified incubator at 37°C with an atmosphere containing 5% CO₂.

Quantification of MGMT mRNA by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Quantification of the MGMT gene expression was performed via the RT-qPCR method as described previously (27). Complementary DNA was synthesized from 1 μ g total RNA isolated from five melanoma cell lines using the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA, USA) with a random primer, 40 units M-MLV reverse transcriptase (both from Invitrogen; Thermo Fisher Scientific, Inc.), 0.5 mM dNTP, 24 units RNase inhibitor (both from Takara Bio, Inc., Otsu, Japan), 10 μ M DTT (Invitrogen; Thermo Fisher Scientific, Inc.) and 5X RT buffer at 37°C for 60 min. The qPCR mixture was prepared using a TaqMan Universal Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.), 120 nM of each primer and 2.5 μ l of each cDNA sample. The primers were as follows: MGMT, forward, 5'-CCT GGC TGA ATG CCT ATT TCC-3' and reverse, 5'-GAT GAG GAT GGG GAC AGG ATT-3'; and

200 nM probe, 5'-CGA GCA GTG GGA GGA GCA ATG AGA-3'. The qPCR conditions were as follows: initial denaturation at 95°C for 10 min; denaturation 45 cycles at 95°C for 30 sec, annealing at 60°C for 30 sec, elongation at 72°C for 30 sec; and final extension, 72°C for 7 min, using a real-time PCR system (ABI PRISM 7900HT Sequence Detection System; Applied Biosystems; Thermo Fisher Scientific, Inc.) as previously reported (28). Glyceraldehyde-3-phosphatase dehydrogenase (*GADPH*) mRNA expression levels were employed as the quantitative internal control. Standard curves for *MGMT* and *GADPH* mRNA were generated using 10-fold serially diluted standard plasmid clones (pCR2.1-TOPO TA Vector; Thermo Fisher Scientific, Inc.) containing MGMT or GAPDH PCR products as templates as previously reported (28,29). The amount of each mRNA expression level was calculated from the relevant standard curve. For precise quantification, the *MGMT* mRNA expression levels of each cell line was normalized based on expression of *GADPH*. The expression levels were calculated using the equations by comparing the threshold cycles (30).

Growth inhibitory effect. The growth inhibition of malignant melanoma cells by TMZ alone, or a combination of TMZ and IFN- β was evaluated by counting the number of cells as previously reported (27). Each well was seeded with 1x10⁴ cells in 24-well plates and cultured for 24 h at 37°C. The cells were incubated with fresh medium containing 0.1-1,000 μ M of TMZ, or medium containing a combination of 0.1, 1, 10, 100 or 1,000 μ M of TMZ and 10 IU/ml of IFN- β ; the cells were cultured for 72 h at 37°C. The latter incubation condition (10 IU/ml of IFN- β) was selected as this condition represents a clinically relevant concentration of IFN- β (31,32). For the depletion of the MGMT activity, 10 μ M of O⁶-BG was added to the cells at 1 h prior to drug treatment (15). The cells were trypsinized at room temperature for 5 min with 0.25% Trypsin-EDTA solution (Invitrogen; Thermo Fisher Scientific, Inc.) and counted using a ZI Coulter Counter® (Beckman Coulter, Inc., Brea, CA, USA). The experiments were repeated at least four times at each concentration.

Cell cycle distribution analysis. The cells were plated at 2x10⁵ cells in a 6-well plate and incubated for 24 h until attachment to the plate was observed. Following drug treatment, the cells were harvested using a trypsin-EDTA solution at 8 and 24 h, and fixed in ice-cold 70% ethanol overnight. The fixed cells were treated with 0.5% RNase A (Roche Diagnostics GmbH, Mannheim, Germany) and stained with 1 μ g/ml propidium iodide (PI) for 30 min at room temperature. The fluorescence was measured with a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) at a wavelength of 610 nm (FL3). The DNA histograms were analyzed using FlowJo software (version 10.2) (FlowJo LLC, Ashland, OR, USA).

Determination of apoptotic cells. Based on the results for the growth inhibitory and *MGMT* mRNA expression analyses, A375 and CRL-1579 cells were employed in the further experiments. Apoptosis was detected by dual staining at room temperature for 10 min with Annexin V and Propidium Iodide (PI) using Annexin V Alexa Fluor® 488 conjugate

(Thermo Fisher Scientific, Inc.) and PI (Miltenyi Biotec, Inc., Cambridge, CA, USA). A375 and CRL-1579 cells were treated with TMZ alone, or with a combination of TMZ and IFN- β for 24, 66 and 72 h at room temperature; adherent and floating cells were then harvested and washed with cold PBS(-) and re-suspended in 1X binding buffer (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Cells (1×10^6) were treated with 5 μ l of Annexin V Alexa Fluor 488 conjugate and 10 μ l of PI solution, and immediately analyzed by flow cytometry according to the manufacturer's protocols. Flow cytometry was performed with a FACSCalibur flow cytometer and apoptosis was analyzed using Flowjo software.

Western blot analysis. Soluble protein lysates of subconfluent melanoma cells were obtained using radioimmunoprecipitation buffer (Wako Pure Chemical Industries, Ltd.) containing protease inhibitors (Complete Mini, EDTA-free; Roche Diagnostics GmbH) for 20 min on ice. Following centrifugation at $20,613 \times g$ for 1 h at 4°C, the protein content of the separated supernatants was determined by using a Bicinchoninic Acid assay kit (Pierce; Thermo Fisher Scientific, Inc.). The proteins (50 μ g) were loaded and separated by 12.5% polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes for 30 min at 15 V with a Bio-Rad Trans Blot® (both from Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membrane was treated with blocking buffer (1% skimmed milk) for 1 h at room temperature, and subsequently incubated with primary antibodies in fresh blocking buffer for 24 h at 4°C. Following rinsing the membrane with washing buffer (PBS/0.05% Tween-20), the membranes were incubated with a secondary antibody conjugated with horseradish peroxidase (HRP) for 1 h at 37°C. The primary antibodies employed were all specific anti-mouse monoclonal antibodies: Anti-Fas (1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-caspase-3 (1:500), anti-caspase-8 (1:1,000), anti-caspase-9 (1:1,000) (both from Cell Signaling Technology, Inc., Danvers, MA, USA), anti-p53 (1:500), anti-phosphorylated (p)-p53 (1:500), anti-p21 (1:500), anti-B-cell lymphoma 2-associated X protein (Bax; 1:500) (all from Santa Cruz Biotechnology, Inc.), anti-microtubule-associated protein light-chain 3 (LC3) antibody (1:1,000), anti-autophagy regulated gene 5/12 complex (Atg5/Atg12 complex) antibody (1:1,000) (both from Medical & Biological Laboratories Co., Ltd., Aichi, Japan) and β -actin (1:2,000; Wako Pure Chemical Industries, Ltd.). The secondary antibody was an HRP-conjugated anti-mouse IgG (Sigma-Aldrich; Darmstadt, Germany) for 1 h at room temperature. The transferred membrane was washed three times with washing buffer and visualized using LAS-4000 (GE Healthcare, Chicago, IL, USA) following treatment with ECL Prime Western Blotting Detection Reagent according to the manufacturer's protocols (GE Healthcare).

Statistical analysis. All studies were repeated 2-3 times. One-way analysis of variance conducted indicated demonstrated a significant difference between the group and the Tukey-Kramer test was used for multiple comparisons. Data were expressed as the means \pm standard error. $P < 0.05$ was considered to indicate a statistically significant difference. Data analyses were performed using SPSS Statistics version 21.0 (IBM Corporation, Armonk, NY, USA).

Results

Quantitative MGMT mRNA expression of melanoma cell lines. An important mechanism of resistance to methylating agents, including DTIC and TMZ is DNA repair mediated by the damage-reversal suicide enzyme, MGMT (33). The absolute values for MGMT mRNA in A375, G361, MeWo and SK-MEL-28 were 9.8×10^3 copies/ μ g RNA, 7.3×10^3 copies/ μ g RNA, 1.5×10^4 copies/ μ g RNA and 7.8×10^3 copies/ μ g RNA, respectively. In contrast, such expression was not detected in CRL-1579 (data not shown). These findings indicated that the A375, G361, MeWo, and SK-MEL-28 cell lines expressed MGMT, whereas the CRL-1579 cell line did not.

Antitumor efficacy of TMZ, and the combination of TMZ and IFN- β . To assess the anti-tumor effects of drug treatments, we treated the 5 melanoma cell lines with 0-1,000 μ M of TMZ alone, or with 0-1,000 μ M of TMZ plus 10 IU/ml of IFN- β ; cells were cultured for 72 h. The growth of all 5 cell lines was inhibited by TMZ alone, and with a combination of TMZ and IFN- β ; however, the sensitivity of each of the cell lines varied. The cell growth inhibitory effect of TMZ combined with IFN- β , compared with TMZ alone, exhibited a dose-dependent pattern, particularly in A375 and CRL-1579 cells (Fig. 1).

Growth inhibitory effects. The concentration of TMZ applied was set at 10 μ M, as this level of TMZ demonstrated growth inhibition in not only MGMT-proficient cells, but also MGMT-deficient cells (Fig. 2). Additionally, this condition represents a clinically applicable dose of TMZ. MGMT is a key determinant of cellular resistance to methylating agents, such as DTIC and TMZ (27). Based on the aforementioned results for the quantification of MGMT mRNA expression and anti-proliferative efficacy, A375 and CRL-1579 cell lines were selected for further experiments.

O⁶-BG is a modulating agent that induces the inactivation and depletion of MGMT, which is the DNA repair protein, by serving as a substrate and transferring its benzyl group to the active site of MGMT (34). To examine whether the expression of MGMT is responsible for the efficacy of growth inhibition, experiments with A375 and CRL-1579 cells were performed under the specific MGMT inhibitor O⁶-BG pretreatment conditions for the depletion of residual MGMT activity. In A375 cells, addition of O⁶-BG prior to TMZ treatment significantly induced growth inhibition of the melanoma cells compared with TMZ treatment alone. Furthermore, inactivation of MGMT via O⁶-BG significantly sensitized A375 cells to combined treatment with TMZ and IFN- β compared with combined treatment alone. These findings indicated that O⁶-BG potentiated the anti-proliferative effects of TMZ and that IFN- β induced by notable sensitization to TMZ when MGMT was depleted with the specific MGMT inhibitor O⁶-BG in A375 cells. Conversely, in CRL-1579 cells, there was no significant difference in the growth inhibitory effects between TMZ alone, and combined treatment of TMZ and O⁶-BG; however, the addition of IFN- β led to significantly decreased cell viability under pretreatment with O⁶-BG compared with combination treatment of CRL-1579 cells (Fig. 3).

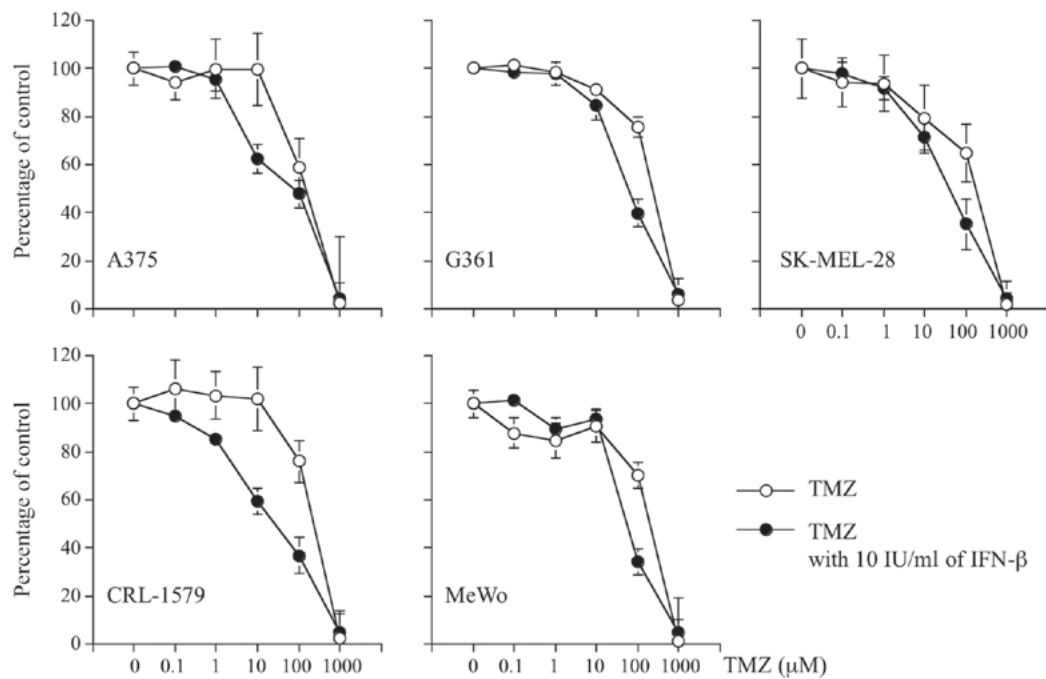


Figure 1. Antiproliferative effects of TMZ and IFN- β on malignant melanoma cell lines. TMZ exerted a cell growth inhibitory effect in all cell lines. IFN- β enhanced the effects of TMZ, particularly in A375 and CRL-1579 cells. TMZ, temozolomide.

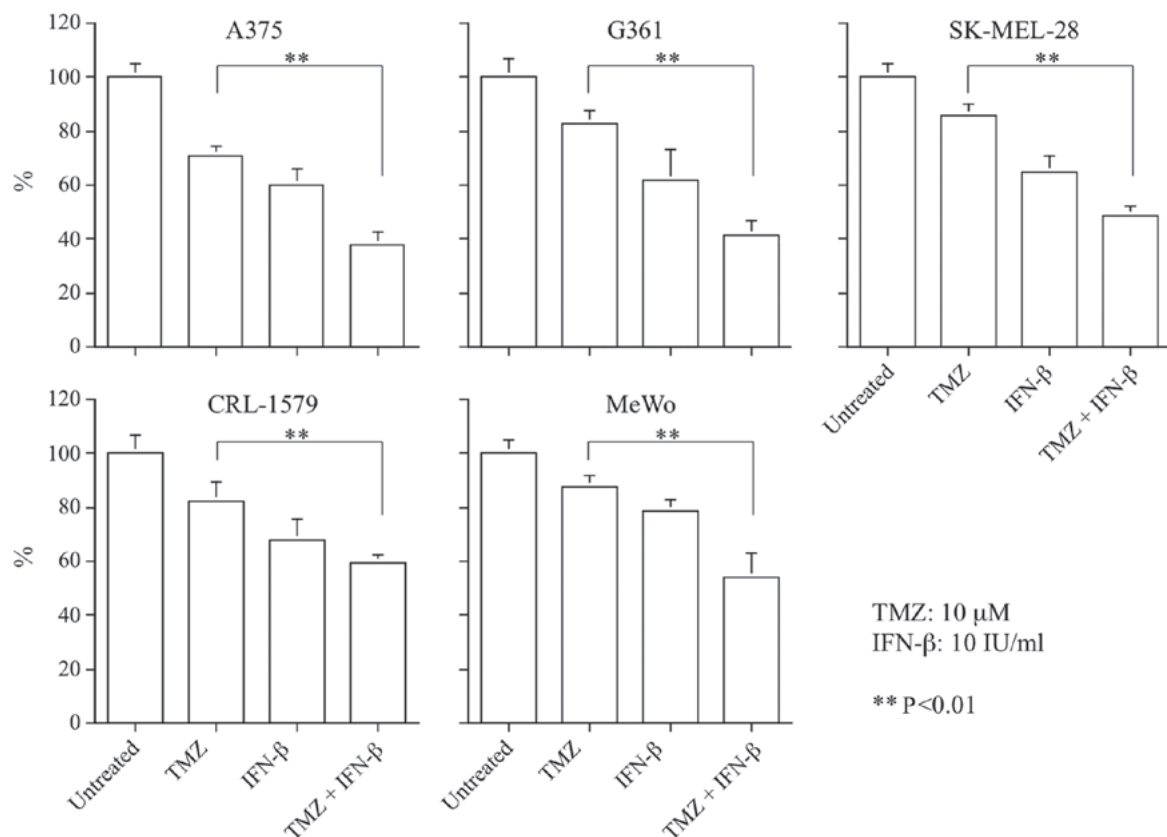


Figure 2. Effects of IFN- β on TMZ-induced growth inhibition. Each well was seeded with 1×10^4 cells in 24-well plates and cultured for 72 h. A375 and CRL-1579 cells exhibited a significant suppression of cell proliferation following combination treatment. Data are presented as the mean \pm standard error. ** $P < 0.05$ vs. IFN- β , interferon- β ; TMZ, temozolomide.

Cell cycle analysis distribution. We performed DNA flow cytometry analysis to investigate whether cell cycle arrest could be induced in malignant melanoma cells by TMZ alone, and

with a combination of TMZ and IFN- β . DNA histograms and the proportions of each cell cycle phase are presented in Fig. 4. We obtained equivalent results from repeating the analyses in

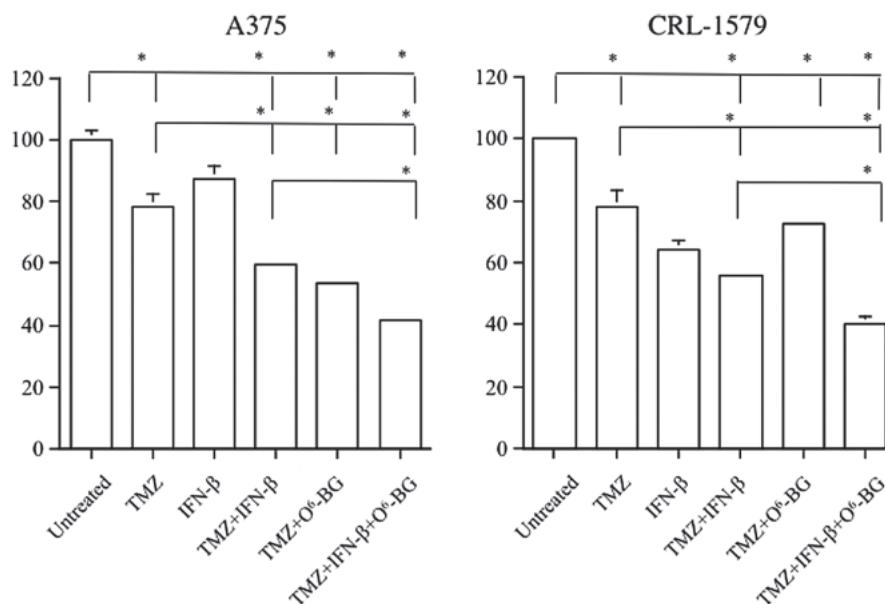


Figure 3. Effects of O⁶-BG. When pretreated with O⁶-BG, IFN-β elicited significant sensitization to TMZ in A375 cells. In CRL-1579 cells, which are MGMT deficient cells, combined treatment TMZ and O⁶-BG in addition to IFN-β led to decreased cell viability. This suggests that IFN-β increased the sensitization effects of TMZ. Data are presented as the mean ± standard error. *P<0.01. IFN-β, interferon-β; O⁶-benzylguanine; TMZ, temozolomide.

triplicate; however statistical significance was not observed. In A375 cells, TMZ alone and combination treatment notably increased the number of cells in G₀/G₁ phase and decreased the abundance of G₂/M phase cells at 8 h. Following treatment for 24 h, a combination of TMZ and IFN-β increased the population of S and the G₂/M phase cells compared with the untreated control. In CRL-1579 cells, TMZ alone and combination treatment increased the cell population in G₀/G₁ phase and decreased that in G₂/M phase at 8 h. Following treatment for 24 h, a combination of TMZ and IFN-β increased the abundance of cells in G₂/M and S phase. These findings indicated that TMZ treatment, and a combination of TMZ and IFN-β could induce G₀/G₁ arrest in melanoma cell lines at 8 h; however, the combined treatment induced G₂/M arrest in a pattern which differed from that of TMZ alone, particularly in CRL-1579 cells.

Western blot analysis of cell cycle-associated proteins. We hypothesized that TMZ-induced G₀/G₁ arrest and combined-treatment-induced G₂/M arrest in melanoma cells were associated with alterations in the expression of proteins that serve a key role in cell cycle regulation. p53 and p21 have important roles in cellular growth. Thus, we analyzed protein expression associated with the cell cycle mediated by p53 using western blot analysis (Fig. 5). In A375 cells, the expression levels of p53, and p-p53 were increased after 4 h and were further upregulated after 24 h following treatment with TMZ alone. The p21 expression levels were increased after 8-48 h. Following combined treatment with TMZ and IFN-β, the expression levels of p53, p-p53 and p21 were increased after 4-24 h. Similar results were obtained for CRL-1579 cells; however, p53, p-p53, and p21 were notably induced in CRL-1579 cells than in A375 cells following combined treatment with TMZ and IFN-β at 4 and 8 h. p21 was observed to show maximum expression after 4 or 8 h.

Activation of apoptosis in melanoma cells induced by TMZ alone, and a combination of TMZ and IFN-β. The induction

of apoptosis by TMZ and IFN-β in human melanoma cells was examined by Annexin V/PI double staining and measured using flow cytometry. A375 and CRL-1579 cells were treated with TMZ alone, IFN-β alone, or a combination of TMZ and IFN-β. A375 cells treated with TMZ alone for 72 h exhibited a 14.5% increase in Annexin V/PI staining. Following treatment with a combination of TMZ and IFN-β for 72 h, the staining intensity increased to 38.7% (Fig. 6). Conversely, CRL-1579 cells pretreated with TMZ alone for 72 h exhibited a 27.5% increase in Annexin V/PI positivity. Following treatment with a combination of TMZ and IFN-β for 72 h, 56.3% of the cell were positively stained (Fig. 6).

Protein expression of apoptosis-associated protein is induced by TMZ alone or in combination with IFN-β. We assessed several apoptosis-associated proteins by western blot analysis in order to examine the mechanisms underlying the anti-apoptotic effect of TMZ and IFN-β. The intrinsic mitochondrial pathway associated with apoptosis was analyzed, and the extrinsic apoptotic pathway mediated by Fas was also investigated. In melanoma cells treated with TMZ alone, the expression levels of p53 and p-p53 were activated and the levels of Bax, and caspases-9 and -3 were also increased (Fig. 7A and B). In cells treated with a combination of TMZ and IFN-β, the findings were similar to those for the cells treated with TMZ alone; however, the duration of activation following treatment with TMZ and IFN-β appeared to be earlier compared with TMZ alone. Conversely, the expression of caspases-8 and -3 in melanoma cells treated with a combination of TMZ and IFN-β were upregulated compared with in melanoma cells exposed to TMZ alone after 4 h. This suggests activation of the extrinsic apoptotic pathway mediated by caspase-8 (Fig. 7A and B). Based on these results, IFN-β may synergize with TMZ and the combination treatment may potentiate cell death via the intrinsic mitochondrial and the extrinsic signaling pathways.

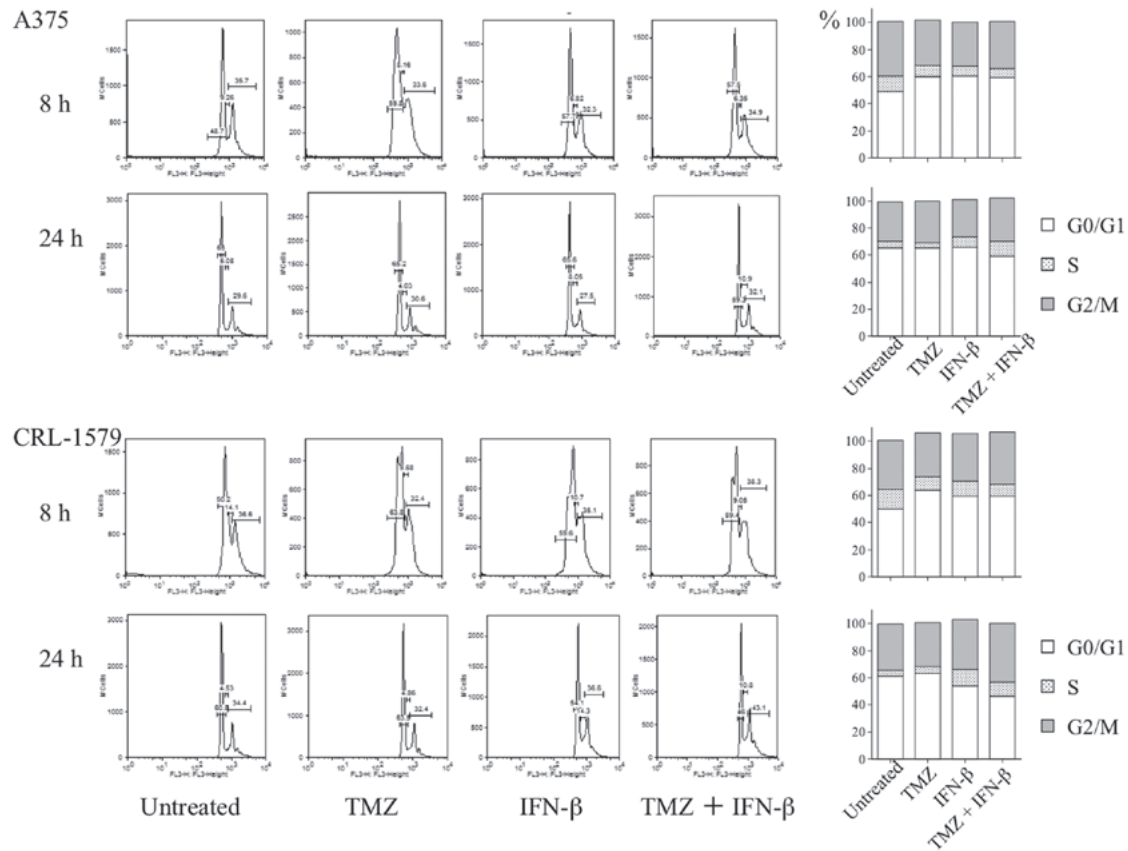


Figure 4. Cell cycle distribution of A375 and CRL-1579 cells treated with TMZ alone, IFN- β alone, of a combination of TMZ and IFN- β for 8 or 24 h. Untreated cells without TMZ alone, IFN- β alone or a combination of TMZ and IFN- β served as the control. The cells were plated at 2×10^5 cells in a 6-well plate. The fluorescence-activated cell sorting data demonstrated an increase of cells in the G₀/G₁ phase following the combination treatment compared with untreated cells at 8 h however, the number of G₂/M cells increased at 24 following combination treatment, indicating that the antitumor efficacy of the combination treatment resulted from the accumulation of cells in the G₂/M phase. IFN- β , interferon- β ; O⁶-benzylguanine; TMZ, temozolomide.

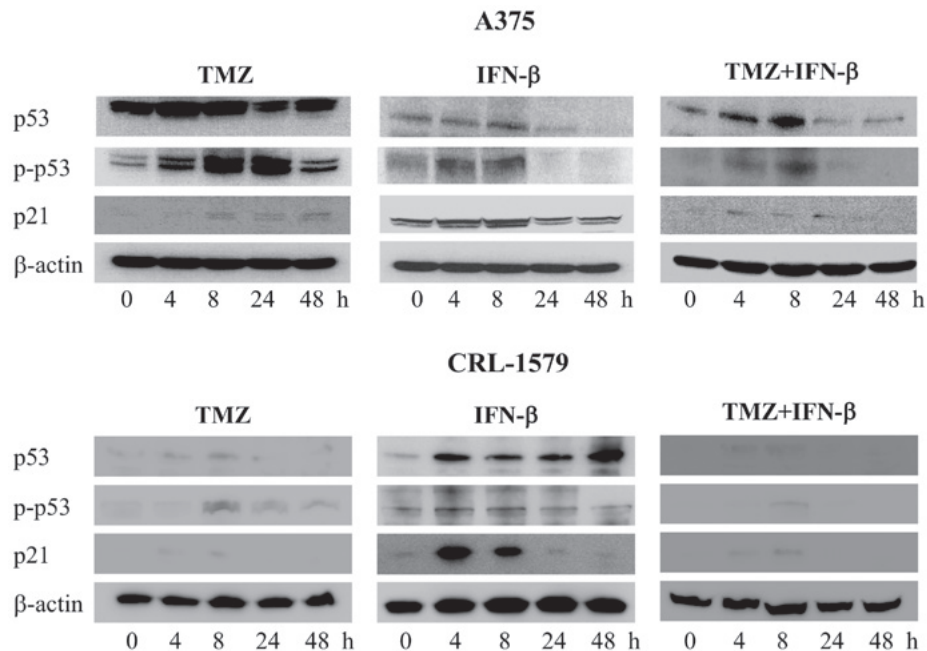


Figure 5. Western blot analysis of proteins associated with the cell cycle. p53, p-p53, and p21 were modulated in response to TMZ alone, IFN- β alone, or combined treatment of TMZ and IFN- β in A375 and CRL-1579 cells. The protein expressions increased after 4 h. IFN- β , interferon- β ; TMZ, temozolomide.

Protein expression associated with autophagy is induced by TMZ alone or in combination with IFN- β . We examined

whether TMZ alone or in combination with IFN- β can induce autophagy in melanoma cells. The expression levels of LC3

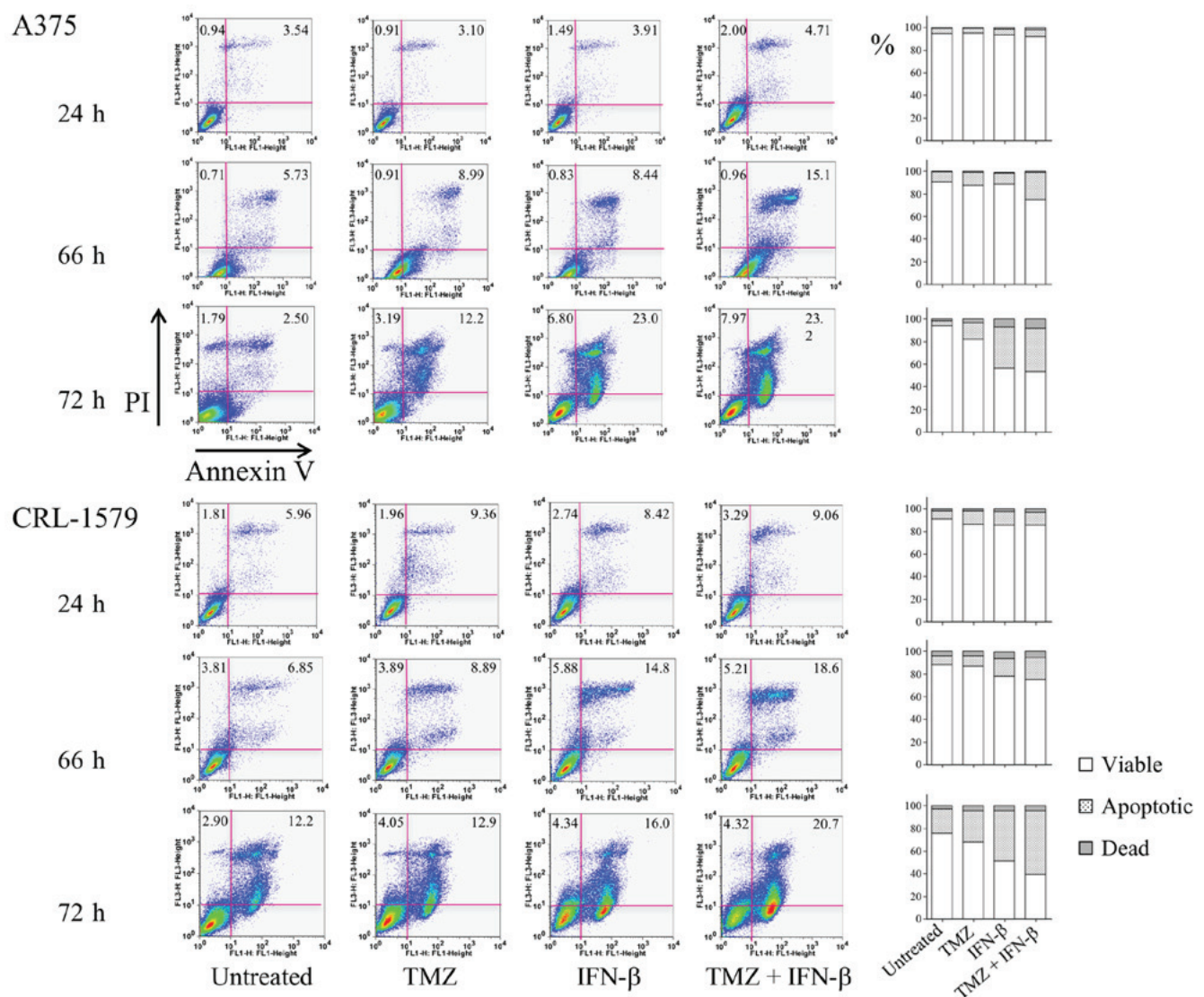


Figure 6. IFN- β induced the apoptosis of A375 and CRL-1579 cells. Combined treatment induced increases in Annexin V- and Annexin V/PI-staining of A375 and CRL-1579 cells. 1×10^6 cells were treated with $5 \mu\text{l}$ of Annexin V Alexa Fluor[®] 488 conjugate and $10 \mu\text{l}$ of PI solution. The percentages of Annexin V-positive Annexin V/PI-positive cells were presented at in the bottom right and top right quadrants, respectively. IFN- β , interferon- β ; PI, propidium iodide; TMZ, temozolomide.

protein and Atg5/Atg12 complex proteins were elevated following TMZ treatment at 4 h in A375 and CRL-1579 cells. These protein levels were clearly increased and activated relatively soon after treatment with TMZ and IFN- β in A375 and CRL-1579 cells (Fig. 8). These findings indicated that the induction of autophagy by TMZ and IFN- β may promote this process in melanoma cells.

Discussion

In the present study, we found that the antiproliferative properties in melanoma cells may be ascribed to inhibition of cell growth, and the induction of apoptosis and autophagy.

The effects of methylating agents have been hypothesized to be dependent on tumoral expression of MGMT. Overexpression of MGMT protects against alkylation-induced cell death; low expression of MGMT is correlated with a high likelihood of a response to methylating agents (12,35). Melanomas tend to express low levels of MGMT (36,37), which could explain why

melanomas respond to methylating drugs, including DTIC and TMZ, but not to other anticancer drugs. MGMT can determine the clinical outcome in melanoma therapy with methylating drugs; however, conflicting data have been reported (33). No significant association was observed between MGMT immunorexpression and the response to TMZ (13,14). The present study demonstrated that A375 cells were more sensitized to TMZ than CRL-1579 cells following depletion of MGMT with O⁶-BG. In addition, these two cell lines exhibited a notably increased sensitivity to combined treatment with TMZ and IFN- β than TMZ alone. These findings were consistent with those reported Roos *et al* (15), that is, IFN- β increased sensitization effects of cells to TMZ, when depleting MGMT with O⁶-BG. The addition of IFN- β led to decreases in cell viability when depleted with O⁶-BG; analysis of MGMT-deficient cells suggested that O⁶-BG may have increased cell death sensitivity (38), and that IFN- β did not affect MGMT (15).

The G₂/M checkpoint serves an important role in the action of TMZ in melanomas (39,40). We revealed that IFN- β

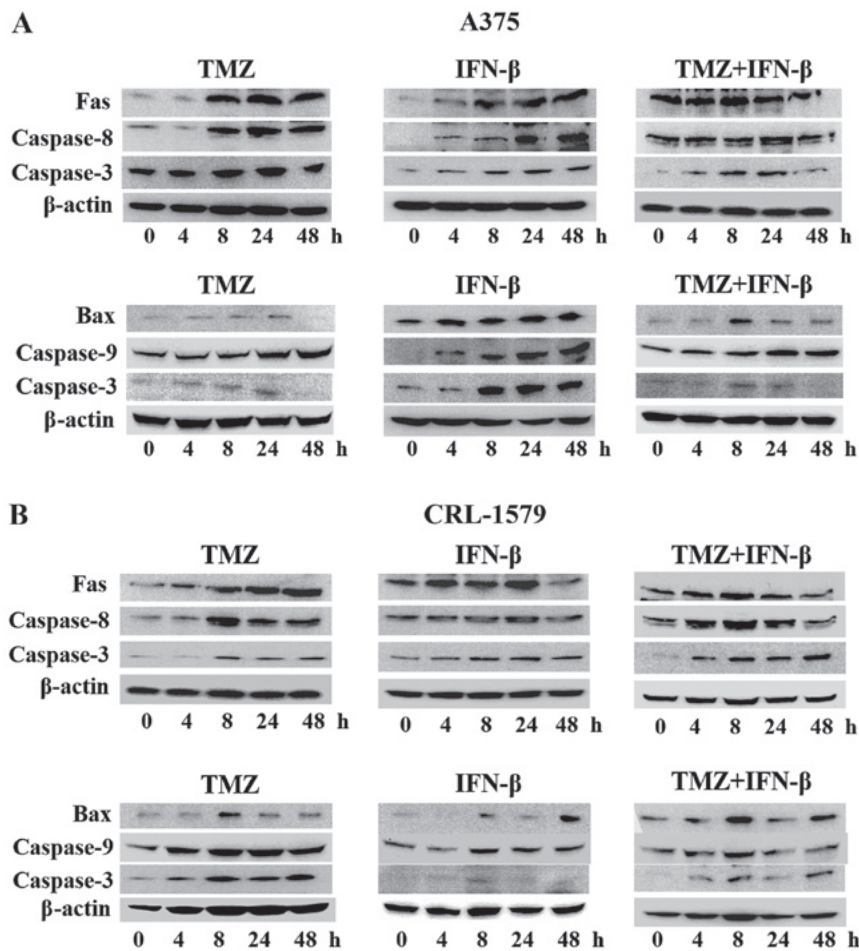


Figure 7. Western blot analysis of proteins associated with apoptosis in A375 and CRL-1579 cells. TMZ treatment activated the intrinsic mitochondrial and the extrinsic signaling pathway in (A) A375 and (B) CRL-1579 cells. In particular, combined treatment with TMZ and IFN- β activated the intrinsic mitochondrial and extrinsic signaling pathways. Bax, B-cell lymphoma-2-associated X protein; IFN- β , interferon- β ; TMZ, temozolomide.

sensitized melanoma cells to TMZ to induce G₂/M arrest. In addition, combination treatment of TMZ and IFN- β was associated with alterations in the expression of proteins that serve a key role in the regulation of the cell cycle.

The biological outcomes of p53 activity include the inhibition of cell cycle progression, senescence, differentiation, acceleration of DNA repair and apoptosis (41). p21 and cyclin-dependent kinase inhibitor 1 are activated by p53 and induces cell cycle arrest in G₀/G₁ phase. In the present study, the expression levels of p21 as investigated by western blot analysis increased following combined treatment of TMZ and IFN- β treatment of A375 and CRL-1579 cells. The data suggested that one of the mechanisms underlying the antitumor effects this particular treatment combination is cell cycle arrest in melanoma cells. p53 inhibits cell cycle progression and has been demonstrated to be functionally inactivated in a variety of human cancers (42). IFN- α/β signaling affects the p53 responses in tumor suppression and antiviral defense (43). The sensitization effect of IFN- β in TMZ-treated melanoma cells is dependent on p53 (15), in which p21 is directly activated (44). In addition to growth inhibition, G₂/M cell cycle arrest as a result of p21 activation following treatment with TMZ plus IFN- β was observed in the present study. As p21 has been observed to be associated with the induction of apoptosis (45,46), upregulation of p21 in melanoma cells may affect the induction of apoptosis.

IFN- β induced apoptosis, which was linked to the activation of Bax gene expression (47,48).

The present study investigated apoptotic cell death via Annexin V/PI staining. There are several distinct processes associated with cell death which include not only apoptosis and necrosis, but also necroptosis, autophagy and ferroptosis (49). In the present study, there appeared to be a discrepancy between the results of growth inhibition and apoptosis analyses. Different methods were conducted and different cell process were investigated; however, an antitumor effect was observed following combination treatment. We also investigated numerous apoptosis-related proteins by western blot analysis. The proportion of Annexin V-positive melanoma cells increased irrespective of the treatment in a time-dependent manner; however, the proportion of apoptotic cells were higher for combination treatment with TMZ and IFN- β than TMZ alone in A375 and CRL-1579 cells. Compared with A375 cells, the number of apoptotic cells was notable increased in CRL-1579 cells following combined treatment. IFN- β in combination with TMZ led to a high degree of early apoptotic responses, particularly in CRL-1579 cells by flow cytometric analysis. A key regulator of apoptosis is the mitochondrial intrinsic signaling pathway and another is the Fas/cluster of differentiation 95/Apo-1 extrinsic signaling pathway. In the present study, p53 and its downstream effector Bax, were

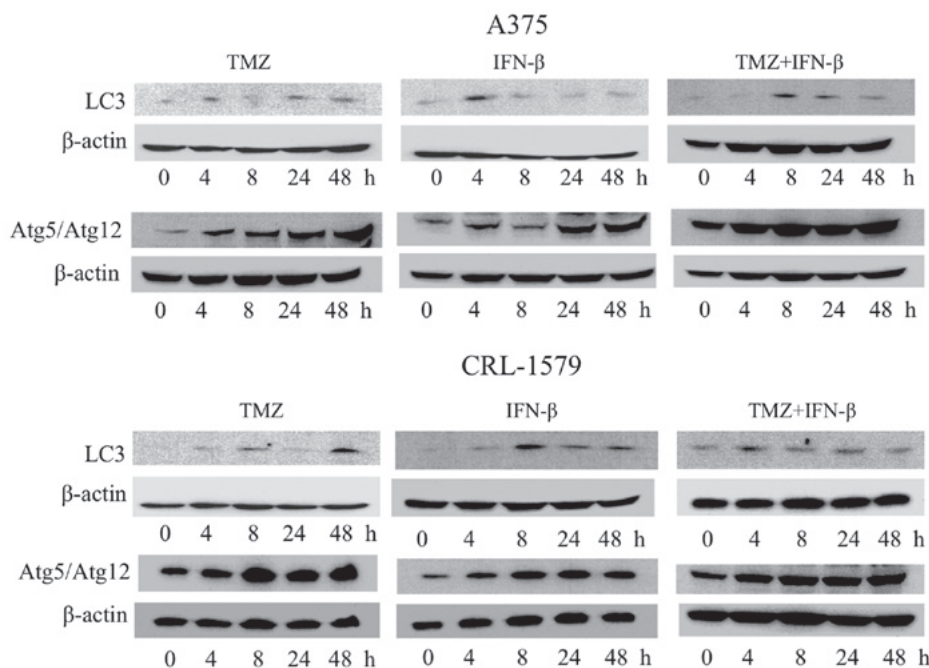


Figure 8. Western blot analysis of proteins associated with autophagy. Expression of the LC3 I and Atg5/Atg12 complex proteins were notably increased and activated following treatment with TMZ, or the combination of TMZ and IFN- β in A375 and CRL-1579 cells. Atg5/Atg12, autophagy regulated gene 5/12 complex; LC, microtubule-associated protein light-chain 3; IFN- β , interferon- β ; TMZ, temozolomide.

upregulated in A375 and CRL-1579 cells treated with TMZ alone. Notable increases in apoptosis were observed following the combined treatment of TMZ + IFN- β . Regarding caspases, we reported increases in the expression of caspases-9 and -3. During the investigation of the intrinsic and extrinsic pathways, the expression of caspase-3 differed, that is, the expression of caspase-3 was upregulated in studying the extrinsic and intrinsic pathways following combination treatment of CRL-1579, and that caspase-3 was upregulated in A375 cells. Unknown factors may have affected caspase-3 or cell variation may be a contributing cause for differing observations; however, the underlying mechanism requires further investigation. Melanomas express numerous extrinsic death receptors, Fas, tumor necrosis factor (TNF) and TNF-related apoptosis-inducing ligand (7). Activation of the apoptotic pathway may occur via Fas-L/FasR and activation of downstream caspase-8 (50,51). In the present study, a combination of TMZ and IFN- β induced Fas expression and its proximal downstream caspase-8 expression, thereby activating the Fas/CD95/Apo-1 extrinsic apoptotic signaling pathway. Roos *et al* (15) have reported that the induction of Fas/CD95/Apo-1 by TMZ and the upregulation of procaspase-8 are required for the sensitization observed during combination therapy.

We demonstrated that malignant melanoma cells undergo apoptosis following the treatments with TMZ and IFN- β . In addition, the present study revealed that CRL-1579 cells exhibited a higher degree of apoptosis compared with A375 cells, which indicated that alkylation-induced cell death may be largely attributable to apoptosis and O⁶-methyl-guanine (O⁶MeG) acted as an inducer in the toxic response of TMZ (36,52,53). The apoptotic process investigated in our study required p53, and O⁶MeG may lead to the apoptosis of A375 and CRL-1579 cells with dependence on p53. These findings suggested that IFN- β may promote the apoptosis induced by TMZ in melanoma cells.

Recent evidence has demonstrated that autophagy can often occur with apoptosis during the process of programmed cell death (30). In the present study, it was observed that TMZ alone and in combination with IFN- β induced not only apoptosis, but also autophagy in melanoma cells. Therefore, IFN- β may markedly induce autophagy when combined with TMZ. Autophagy was originally designated as a process of protein recycling (54). Autophagosome nucleation is mediated by Beclin 1 (Atg6). Subsequently, the Atg5/Atg12 complex and LC3 (Atg8) are required for the elongation of autophagosomes (55). The abundance of LC3-II is increased via the conversion of LC3-I (56). Autophagy serves complex roles in cancer, which can promote or inhibit tumorigenesis (54). Functional crosstalk exists between autophagy and apoptosis (57), and autophagy can occur with apoptosis in the process of programmed cell death (53). Autophagic degradation of active caspase-8 has been associated with the crosstalk between autophagy and apoptosis (58). IFN- β also regulates the adaptive immune response and the innate immune response by inducing C-X-C motif chemokine ligand 10 or Toll-like receptor 3 (59). These processes require further investigation and the effects of IFN-dependent innate immunity in cancer are to be determined in the future.

The results of the present study suggested that the clinical therapeutic efficacy of TMZ may be enhanced by the combined treatment of TMZ and IFN- β in malignant melanomas. Combination treatment of TMZ and IFN- β suppressed cell proliferation, and enhanced apoptosis and autophagy in melanoma cells. The present study did not investigate the chemoresistance of melanoma cells treated with TMZ and IFN- β ; the underlying mechanism remains unknown. On the contrary, our data demonstrated that one of the possible immunomodulatory effects of IFN- β may be that IFN- β acts as a sensitizer of malignant melanomas to enhance the toxicity of TMZ. Experiments using TMZ and IFN- β were conducted

in the present study as it has been reported that IFN- β exerts notable inhibitory effects on melanoma cells to IFN- α (60-62). Thus, this treatment may be applied in the treatment of melanoma; however, future studies should be conducted.

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Availability of data and materials

All data analysed during this study are included in this article.

Authors' contributions

KM and ES performed the experimental design, most of the experiments and analysis, drafted the manuscript. HH was involved in the conception and design of the study, analyzed the data and contributed to the writing of the manuscript. YO (Ochiai) and TH also conducted experiments and analyzed the data. YO (Okamoto), TU, TN and SA supervised the study and proofread the manuscript. AY contributed to the writing of the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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