DOCTORAL THESIS

Oral recombinant methioninase increases TRAIL receptor-2 expression to regress pancreatic cancer in combination with agonist tigatuzumab in an orthotopic mouse model

(経口 recombinant methioninase は膵癌同所移植マウスの TRAIL レセプター2 の発現を増加させ，tugatuzumab との併用で腫瘍を縮小させる)

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Oral recombinant methioninase increases TRAIL receptor-2 expression to regress pancreatic cancer in combination with agonist tigatuzumab in an orthotopic mouse model

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ABSTRACT

Methionine addiction is a fundamental and general hallmark of cancer. Gene expression analysis showed that methionine restriction (MR) of methionine-addicted cancer cells increases TNF-related apoptosis-induced ligand receptor-2 (TRAIL-R2) expression. Here, we determined the effects of MR on TRAIL-R2 targeted therapy in pancreatic cancer by the TRAIL-R2 agonist tigatuzumab. Human pancreatic cancer cell lines were cultured in control or methionine-free medium. The effects of MR on TRAIL-R2 expression and sensitivity to tigatuzumab were evaluated *in vitro*. An orthotopic pancreatic cancer mouse model was established to evaluate the efficacy of MR using oral recombinant methioninase (o-rMETase), and the efficacy of tigatuzumab and their combination. MR enabled tigatuzumab-induced apoptosis, by increasing TRAIL-R2 expression in pancreatic cancer cells *in vitro*. The protein expression level of the melanoma-associated antigen MAGED2, which reduces TRAIL-R2 expression, was decreased by MR. In the orthotopic pancreatic cancer mouse model, o-rMETase increased TRAIL-R2 expression level in the tumors and enabled the antitumor efficacy of tigatuzumab. MR, effected by o-rMETase, enabled the efficacy of the TRAIL-R2 agonist tigatuzumab by increasing TRAIL-R2 expression in pancreatic cancer. Our results suggest that o-rMETase has clinical potential for treating pancreatic cancer.

1. Introduction

Pancreatic cancer is one of the most lethal cancers in the world. Even now, the 5-year survival rate is only approximately 9% [1]. Only 15%–20% of patients are eligible to undergo potentially curative resection since most tumors are diagnosed with locally advanced disease and/or distant metastases [2]. Even with curative-intent surgery, 80% of the patients recur and die of their disease [2]. In recent years, new chemotherapies, such as nanoparticle albumin-based paclitaxel and FOLFIRINOX, have been approved for further development [3,4]. However, the prognosis remains especially poor, thereby development of new effective treatment is needed.

Cancer cells are methionine (MET) addicted as they have an elevated requirement for MET due to methionine overuse for elevated aberrant transmethylation reaction [5–9]. Methionine addiction was discovered in our laboratory [5] and is a fundamental and general hallmark of cancer. Methionine restriction (MR) induces cell cycle arrest in S/G_2 phase and apoptosis in cancer cells *in vitro* and suppress tumor growth *in*

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vivo [10,11]. In addition, MR enhances the efficacy of cytotoxic chemotherapy drugs by S/G_2-phase cell cycle trap of the cancer cells [12]. Methionine addiction is due to overuse of methionine by cancer cells which is due to excess and aberrant methylation reactions [9,13]. This altered metabolism of cancer cells is termed the Hoffman effect [14], analogous but stronger than the Warburg effect [15]. Methionine-independent revertants of methionine-addicted cancer cells have lost many properties of malignancy [6], including elevated transmethylation [16], which proves that elevated transmethylation is the basis of methionine addiction and perhaps cancer itself.

Recombinant methioninase (rMETase), a methionine-degrading enzyme, has anti-tumor efficacy *in vitro* and *in vivo* [17]. Previously we have reported the efficacy of rMETase in chemotherapy-resistant tumors, including patient-derived orthotopic xenograft (PDOX) mouse models [18,19]. In 2017, we observed the totally unexpressed result that rMETase was as effective by oral dosing as i.p. or i.v. administration [18].

Tumor necrosis factor (TNF)-related apoptosis-induced ligand (TRAIL) is a member of the TNF superfamily. TRAIL induces apoptosis in cancer cells via intrinsic and extrinsic apoptotic pathways by binding the proapoptotic death receptors, TRAIL-R1/DR4 and TRAIL-R2/DR5, and activating procaspase-3/-10 [20,21]. TRAIL-R1 and TRAIL-R2 expression are detected with high frequency in cancer cell lines and clinical tumor specimens compared to normal tissues [22,23]. Thus, TRAIL agonists are expected to provide tumor-specific treatment. Some clinical trials have demonstrated safety and tolerability of TRAIL agonists; however, only small therapeutic benefits have been observed [24,25]. Previous studies showed that expression of TRAIL-R2 was upregulated in some types of tumor cells under the condition of dietary MR [26,27] and MR enhanced the efficacy of a TRAIL-R2 agonist in an orthotopic mouse model of triple-negative breast cancer [25–28]. However, this study used only methionine-free diet to effect MR and could not arrest tumor growth.

The present study used oral dosing of rMETase (o-rMETase) to effect MR along with the TRAIL agonist tigatuzumab and could regress pancreatic cancer in an orthotopic mouse model.

2. Material and Methods

2.1. Cell culture

The MIA PaCa-2 and BxPC-3 human pancreatic cancer cell lines and Hs27 human foreskin fibroblast cell line were used for *in vitro* experiments. MIA PaCa-2 was stably transduced to express red fluorescent protein (RFP) as previously described and used for *in vivo* experiments [29]. Cells were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 100 IU/ml penicillin/streptomycin. Methionine-free DMEM medium, MET(−), was used to closely matched the original medium containing L-methionine.

2.2. Reagents

Tigatuzumab was kindly supplied by Daiichi Sankyo Co., Ltd. (Tokyo, Japan). Tigatuzumab needs cross-linkage with anti-human IgG to exert its anti-tumor efficacy *in vitro*, and thus tigatuzumab was mixed with the same quantity of goat anti-human IgG (2040-01, Southern Biotech, Birmingham, AL) just before treatment *in vitro*. Gemcitabine was generously supplied by Dr. Sant P. Chawla.

2.3. Drug sensitivity assay

Assays were performed using the colorimetric WST-8 reagent (Dojindo Laboratory, Kumamoto, Japan). Cells were cultured in 96 well plates (3×10^3 cells/well) in MET(+) medium overnight. The next day, cells were treated with tigatuzumab along with anti-human IgG at concentrations between 0.05 μg/ml and 50 μg/ml. Cell viability was measured after 48 h of drug treatment. Drug sensitivity curves and IC_{50} values were calculated using ImageJ ver. 1.52 (National Institutes of Health, Bethesda, MD, USA) and Microsoft Excel for Mac 2016 (Microsoft, Redmond, WA, USA).

2.4. Cytotoxic assay under methionine restriction of pancreatic cancer cells in vitro

Cytotoxicity assays were performed using the Cytotoxicity LDH Assay Kit-WST (Dojindo Laboratory, Japan). Cells were cultured in 96 well plates (1×10^4 cells/well) in MET(−) medium overnight and the medium was changed the next day. Cytotoxicity and cell proliferation were measured after 24, 48 and 72 h of the medium change. Experiments were performed three times, each in triplicate.

2.5. Efficacy of methionine restriction in combination with tigatuzumab

Cells were cultured in 96 well plates (3×10^3 cells/well) in MET(+) medium or MET(−) overnight. The next day, MIA PaCa-2 cells were treated with vehicle (PBS), gemcitabine (1.8 μM) or tigatuzumab with anti-human IgG (cross-linked tigatuzumab) (3 μg/ml), while BxPC-3 cells were treated with vehicle (PBS), gemcitabine (3 μM) or cross-linked tigatuzumab (8 μg/ml). Cell viability was measured after 48 h of drug treatment using the colorimetric WST-8 reagent. Cell viability was expressed as the percentage of viable cells compared to control vehicle-treated cells in MET(+) medium. Experiments were performed three times, each in quintuplicate.

2.6. Caspase-3/7 activity assay

Cells were cultured in 96 well plates (2×10^4 cells/well) in MET(+) or MET(−) medium overnight. The next day, MIA PaCa-2 cells were treated with vehicle (PBS), gemcitabine (1.8 μM) or cross-linked tigatuzumab (3 μg/ml), while BxPC-3 cells were treated with vehicle (PBS), gemcitabine (3 μM) or cross-linked tigatuzumab (8 μg/ml) in MET(+) or MET(−) medium. Caspase 3/7 activity was measured after 5 h of drug treatment using the Caspase-Glo 3/7 Assay System (Promega, Madison, WI, USA). Experiments were performed three times, each in triplicate.

2.7. Immunoblotting

For the *in vitro* apoptosis assay, the cells were cultured in 6 wells plates in MET(+) medium overnight. The next day, the wells were washed PBS and added MET(+) or MET(−) medium. After 48 h, MIA PaCa-2 cells were treated with vehicle (PBS), gemcitabine (1.8 μM) or cross-linked tigatuzumab (3 μg/ml), while BxPC-3 cells were treated with vehicle (PBS), gemcitabine (3 μM) or cross-linked tigatuzumab (8 μg/ml). The cells then were lysed and protein extracted after 5-h treatment. For the *in vitro* TRAIL-R2 and MAGED2 expression assays, the cells were cultured in 6 wells plates in MET(+) medium overnight. The next day, the wells were washed PBS and MIA PaCa-2 and Hs27 cells were cultured for 48 h and BxPC-3 cells were cultured for 24 h in MET(+) or MET(−) medium. Cells were also cultured in MET(+) medium along with 0.1 or 1 U/ml rMETase as methionine restriction. Whole-cell lysates and extracted proteins from xenograft tumor sample were obtained. The cells were washed three times with cold PBS and lysed in Pierce RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA) with 1% Halt protease inhibitor cocktail (Thermo Fisher Scientific). The tumors were frozen after collection and homogenized in the same buffer. The lysates were centrifuged at 12,000×*g* for 15 min at 4 °C and debris was removed. Each of the protein samples was subjected to immunoblot analysis using an anti-TRAIL-R2 antibody (1:1,000, ab230969, Abcam, Cambridge, UK), anti-MAGED2 (1:5,000, ab236592, Abcam), anti-caspase-3 (1:5,000, ab32351, Abcam) and an anti-PARP antibody (1:1,000, #9532, Cell Signaling Technology, Danvers, MA, USA). Anti-β-actin antibody was used as the internal control (1:15,000, 20536-1-AP,

Proteintech, Rosemont, IL, USA). Immunoreactive proteins were visualized with Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA). The UVP ChemStudio (Analytik Jena US LLC, Upland, CA, USA) was used to detect the signals.

In the *in vivo* experiments, there was a small amount of a lighter protein detected by the anti-β-actin antibody nonspecifically. This band did not interfere with β-actin serving as an internal loading control.

2.8. Real-time PCR

The cells were cultured in 6-well plates in MET(+) medium overnight. The next day, the wells were washed with PBS and the cells were cultured in MET(+) or MET(−) medium for 48 h. Total RNA was extracted from the cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized from total RNA using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). Quantitative RT-PCR was performed using the predesigned FAM-labelled TaqMan Gene Expression Assay reagents and the 7900HT Fast Real-Time PCR System (Applied Biosystems). Primers for TRAIL-R2 (Hs00366278_m1), MAGED2 (Hs00374760_1) and GAPDH (Hs02786624_g1) were all purchased from Thermo Fisher Scientific. Experiments were performed three times, each in triplicate. A quantitative Ct method was used to compare the RNA expression in samples to that of the control in each experiment. Experiments were performed three times, each in triplicate.

2.9. Animal studies

Athymic *nu/nu* female mice (AntiCancer Inc, San Diego, CA, USA), 4–6 weeks old, were used in this study. All mice were kept in a barrier facility on a high efficacy particulate air (HEPA)-filtered rack under standard conditions of 12 h light/dark cycles. Animal studies were performed with an AntiCancer Institutional Animal Care and Use Committee (IACUC)-protocol specially approved for this study and in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Animals under Assurance Number A3873-1.

2.10. Orthotopic pancreatic cancer xenograft model

After confluence, MIA PaCa-2-RFP human pancreatic cancer cells (2×10^6 cells) were injected subcutaneously into the flanks of nude mice and allowed to engraft and grow over a period of 3–4 weeks. Tumors were then harvested and 30 mm^3 tumor fragments from subcutaneous tumors were sutured to the tail of the pancreas using 7-0 PDS-II surgical sutures (Ethicon, Inc., Somerville, NJ, USA). Upon completion of the operation, the tail of the pancreas was returned to the abdomen, and the incision was closed in one layer using 5-0 PDS-II surgical sutures (Ethicon, Inc) [30,31]. Mice were randomized into four groups when the tumor volume reached 80 mm^3: G1: untreated group; G2: o-rMETase (oral., 100 units/day, daily); G3: tigatuzumab (i.v., 3 mg/kg, weekly, 4 weeks); G4: o-rMETase plus tigatuzumab. In order to treat with tigatuzumab after effecting MR with o-rMETase, tigatuzumab was administered on day 4, 11, 18 and 25. Each group comprised six mice. Treatment doses, routes, and schedules were based on previous reports [32,33]. Tumor volume and body weight were measured once a week using the following formula: tumor volume (mm) = length (mm) × width (mm) × width (mm) × 1/2. Fluorescent tumors were imaged with the FluorVivo version 2.0 (INDEC BioSystems, Santa Clara, CA, USA) fluorescence imaging system every week. All mice were sacrificed on day 28.

2.11. Recombinant methioninase production

Recombinant L-methionine α-deamino-γ-mercapto-methane lyase (rMETase) is a homotetrameric PLP enzyme of 172-kDa molecular mass. An rMETase high-expression clone in *E.coli* was used for rMETase production. The fermentation procedure for host recombinant *Escherichia coli* cells and the purification protocol for rMETase were the same as previously described: rMETase was purified by three different steps using columns including DEAE Sepharose FF (Pharmacia, Uppsala, Sweden), Sephacryl S-200HR (Sigma, St. Louis, MO, USA), and Acti-Clean Etox (Sigma), which is designed for eliminating endotoxin [34] .

2.12. Serum L-methionine (MET) levels

Whole blood was collected by heart puncture from each of five mice in four groups into heparinized tubes on the final date of treatment (day 28). Serum was obtained from the blood by centrifugation at 12,000 rpm in 4 °C for 5 min. Serum MET levels were measured with an HPLC (Hitachi L-6200A Intelligent pump; Hitachi, Ltd., Tokyo, Japan) after derivatization of serum amino acids with the fluoraldehyde reagent OPA as described previously [35].

2.13. H & E staining and immunohistochemistry

Hematoxylin and eosin (H&E) staining and immunohistochemical staining were performed as described [36,37]. Rabbit monoclonal anti-TRAIL-R2 antibody (1:2,000, ab230969, Abcam), rabbit polyclonal anti-MAGED2 antibody (1:500, ab236592, Abcam), rabbit monoclonal anti-cleaved caspase-3 antibody (1:500, ab2302, Abcam) were used.

For immunohistological evaluation, two investigators (Y.T. and S.I.) selected the five most abundant regions of each tissue and counted cleaved caspase-3-positive cells (magnification, 200×) in each of the five regions. In instances where there were discrepancies, a multiheaded microscope was used for consensus.

2.14. Statistical analyses

All statistical analyses were performed with JMP ver. 12.2.0 (SAS Institute, Cary, NC, USA). The Mann-Whitney's *U* test or one-way ANOVA with Tukey post hoc pairwise tests was used to compare between groups for *in vitro* and animal studies. Bar graphs show the mean, and error bars express standard error of the mean. A probability value of $P < 0.05$ was defined as statistically significant.

3. Results

3.1. Methionine restriction sensitizes pancrea\tic cancer cells to tigatuzumab in vitro

We first evaluated the efficacy of tigatuzumab on pancreatic cancer cells in normal methionine medium and IC_{50} values were calculated (Fig. 1A). The IC_{50} dose of tigatuzumab was 2.95 μg/ml for MIA PaCa-2 cells and 8.21 μg/ml for BxPC-3 cells. To evaluate the cytotoxicity of methionine restriction effected by MET-medium on pancreatic cancer cells, the LDH cytotoxicity assay was performed (Fig. 1B). While cell proliferation was suppressed under the condition of MR, cytotoxicity was not increased. These results indicate that MR mainly arrests cell proliferation.

To determine whether MR enhanced the cytotoxicity of tigatuzumab, two human pancreatic cancer cell lines (MIA PaCa-2 and BxPC-3) were grown in MET(+) or MET(−) medium and treated with vehicle, tigatuzumab or gemcitabine. MR alone suppressed the proliferation of two cell lines (Fig. 1C). MR enhanced the toxicity of tigatuzumab in both pancreatic cancer cell lines compared to MET(+) medium. However, MR enhanced toxicity of gemcitabine in only MIA PaCa-2 cells. These results indicate that MR sensitized pancreatic cancer cells to tigatuzumab.

3.2. Methionine restriction increases tigatuzumab-induced caspase activation and apoptosis in pancreatic cancer cells

To determine whether MR enhances caspase activation by

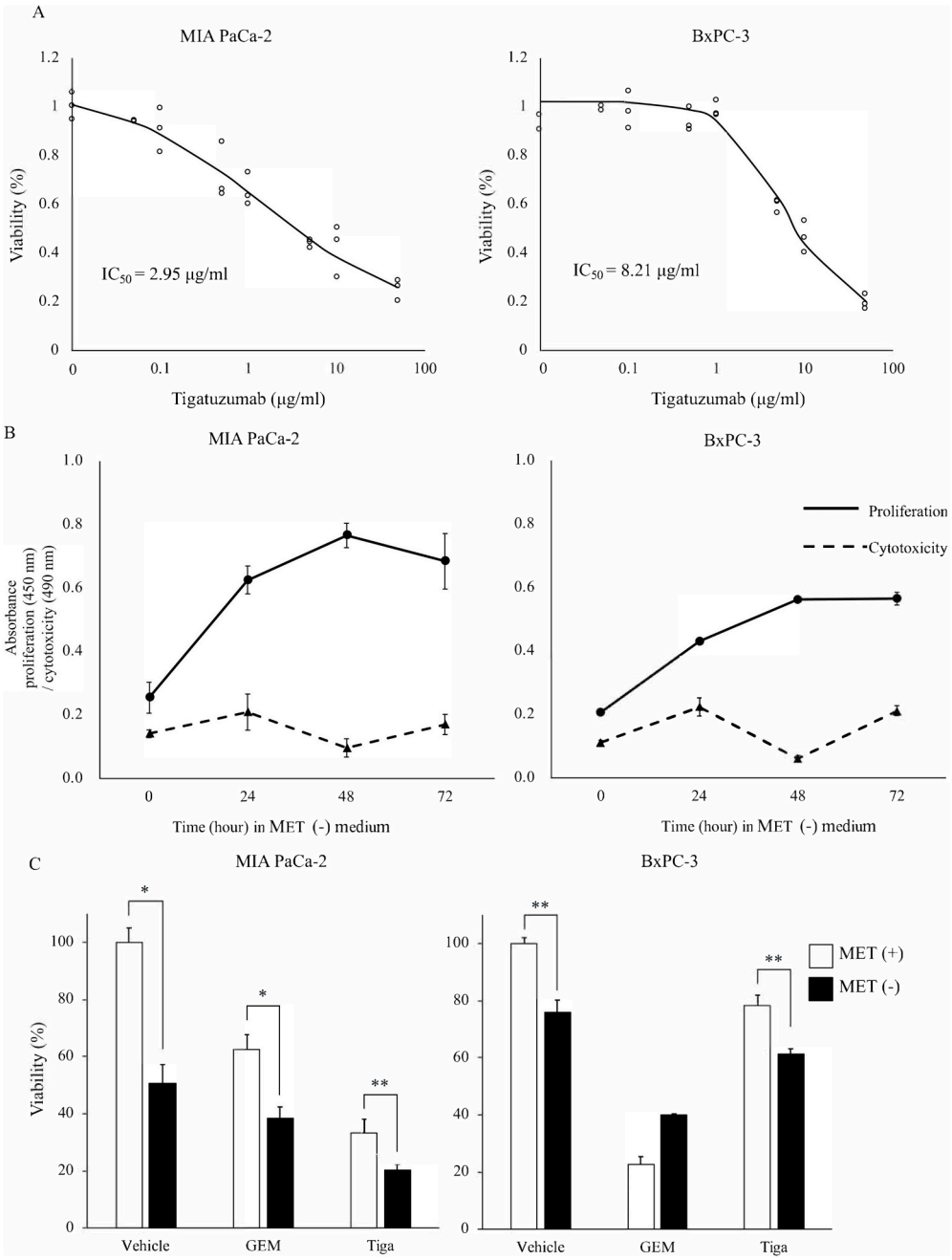

Fig. 1. Methionine restriction enhances tigatuzumab efficacy on pancreatic cancer cells *in vitro*. (A) Sensitivity to tigatuzumab. Cells were cultured in normal medium and treated for 48 h with various concentrations of tigatuzumab. IC_{50} values were calculated using ImageJ 1.52. (B) Cell proliferation assay and LDH cytotoxicity assay under methionine restriction. Cells were cultured in MET(−) medium for 24, 48 and 72 h (mean ± SEM, n = 3). (C) Sensitivity to tigatuzumab and gemcitabine in methionine containing or methionine-free medium. Cells were cultured in normal medium (MET(+)) or methionine-free medium (MET(−)) and treated for 48 h with vehicle, gemcitabine (1.8 μM) or tigatuzumab (3 μg/ml) for MIA PaCa-2 cells and gemcitabine (3 μM) or tigatuzumab (8 μg/ml) for BxPC-3 cells (mean ± SEM, n = 3). *, $p < 0.001$, **, $p < 0.01$.

tigatuzumab, pancreatic cancer cells were grown in MET(+) or MET(−) medium, treated with vehicle, gemcitabine or tigatuzumab, and then analyzed by immunoblotting. MR augmented tigatuzumab-induced proteolytic processing of procaspase-3 as detected by decreased procaspase-3 expression and increased cleaved caspase-3 expression (Fig. 2A). In addition, MR reduced the expression of full-length PARP and/or increased the expression of cleaved PARP. In contrast, MR had little or no effect on gemcitabine-induced PARP cleavage or pro/cleaved caspase-3 processing.

To quantitate the effect of MR for caspase activation, pancreatic cancer cells were grown in MET(+) or MET(−) medium, treated with vehicle, gemcitabine or tigatuzumab, and then analyzed for caspase-3/7 activity level. MR significantly increased the caspase-3/7 activity level of the cells treated with tigatuzumab (MIA PaCa-2; $P < 0.001$, BxPC-3; $P = 0.0018$) (Fig. 2B). In contrast, MR had little or no effect on the caspase-3/7 activity elevation for the cells treated with vehicle or gemcitabine. These findings suggest that MR enabled tigatuzumab-induced caspase activation.

3.3. Methionine restriction increases TRAIL-R2 mRNA and protein expression in pancreatic cancer cells, but not normal fibroblasts

To evaluate the effect of MR on TRAIL-R2 expression, pancreatic cancer cells were grown in MET(+) or MET(−) medium and then analyzed by qRT-PCR and immunoblotting. MR significantly increased the TRAIL-R2 mRNA level in pancreatic cancer cells (MIA PaCa-2; $P = 0.003$, BxPC-3; $P < 0.0001$) (Fig. 3A). MR also increased TRAIL-R2 protein expression (Fig. 3B). These results suggest that MR sensitized pancreatic cancer cells to tigatuzumab by increasing TRAIL-R2 expression. Similarly, rMETase increased TRAIL-R2 protein expression level in pancreatic cancer cells (Fig. 3C). In contrast, MR did not increase TRAIL-R2 protein expression level in Hs27 normal foreskin fibroblasts.

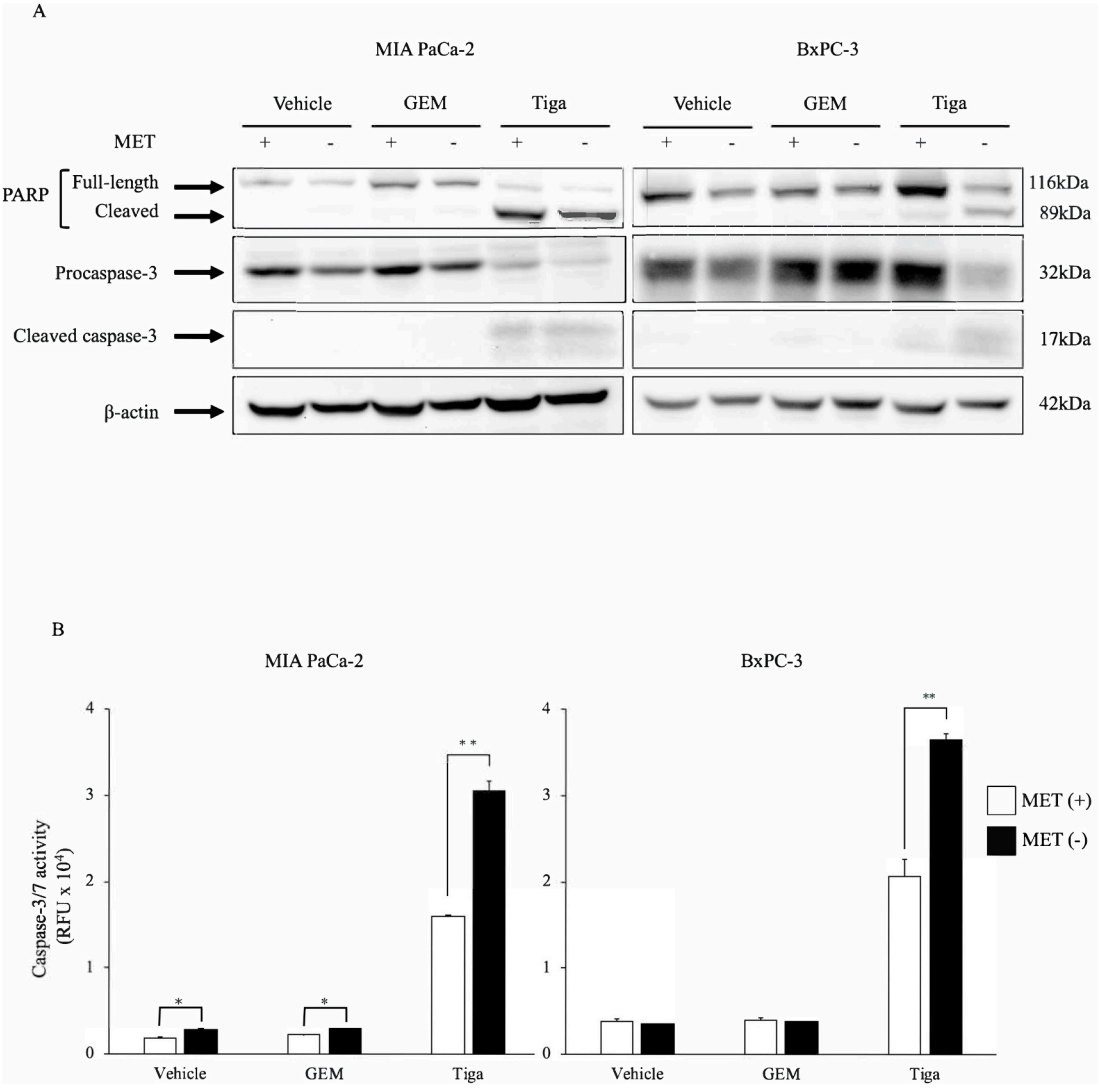

Fig. 2. Methionine restriction increases tigatuzumab-induced caspase activation and apoptosis in pancreatic cancer cells in vitro. (A) Pancreatic cells were grown in MET(+) or MET(−) medium for 48 h and then treated for 5 h with vehicle, gemcitabine (1.8 μM) or tigatuzumab (3 μg/ml) for MIA PaCa-2 cells or gemcitabine (3 μM) and tigatuzumab (8 μg/ml) for BxPC-3 cells. PARP (full length and cleaved form). Procaspase-3 and cleaved caspase-3 were detected by immunoblotting. (B) Cells were plated in 96 well plate (2×10^4 cells/well) in normal medium (MET(+)) or methionine-free medium (MET(−)). Next day, cells were treated with vehicle, gemcitabine (1.8 μM) or tigatuzumab (3 μg/ml) for MIA PaCa-2 cells and gemcitabine (3 μM) or tigatuzumab (8 μg/ml) for BxPC-3 cells for 5 h. Caspase-3/7 activity was assessed by a luminometric assay (mean ± SEM, n = 3). *, $p < 0.01$, **, $p < 0.001$.

3.4. Methionine restriction decreases MAGED2 protein expression without altering mRNA expression in pancreatic cancer cells

Previous studies showed that MAGED2 suppressed TRAIL-R2 expression and silencing MAGED2 cells mimicked the effect of MR [28,38] . Thus, MAGED2 expression under MR was evaluated. RT-PCR revealed that MR did not decrease MAGED2 mRNA levels (MIA PaCa-2; P = 0.888, BxPC-3; P = 0.102) in MET(−) medium (Fig. 4A). However, the expression level of MAGED2 protein was decreased under MR in MET(−) medium (Fig. 4B). These results suggest that MR regulates MAGED2 expression after transcription. Similarly, treatment of pancreatic cancer cells with rMETase decreased MAGED2 protein expression level in pancreatic cancer cells (Fig. 4C). In contrast, MR did not decrease MAGED2 protein expression level in Hs27 normal cells. These results suggest that MR specifically regulates MAGED2 protein expression after transcription in pancreatic cancer cells.

3.5. o-rMETase reduces the serum methionine level and enhances the anti-tumor efficacy of tigatuzumab in a pancreatic-cancer orthotopic model

To examine the antitumor effect of tigatuzumab under MR *in vivo*, a human pancreatic cancer MIA PaCa-2-RFP orthotopic mouse model was used. Mice were randomized into four groups: G1: untreated group; G2: o-rMETase (oral., 100 units/day, daily); G3: tigatuzumab (i.v., 3 mg/kg, weekly); G4: o-rMETase plus tigatuzumab. The combination of o-rMETase plus tigatuzumab was more effective than the control at the end of the treatment period (P = 0.022), and only the combination therapy regressed the tumor volume among the four groups (Fig. 5A and B). There was no significant change of tumor volume, excepts between the control and the combination treatment group (control and tigatuzumab; P = 0.091, tigatuzumab and combination; P = 0.90). There was no significant body weight change between the four groups (Fig. 5C). The serum methionine level of the mice treated with o-rMETase or o-rMETase plus tigatuzumab was significantly decreased (Fig. 5D). Apparent side-effects were not observed in any of four groups. Immunoblotting also showed that the expression level of TRAIL-R2 expression level was

Figure 3

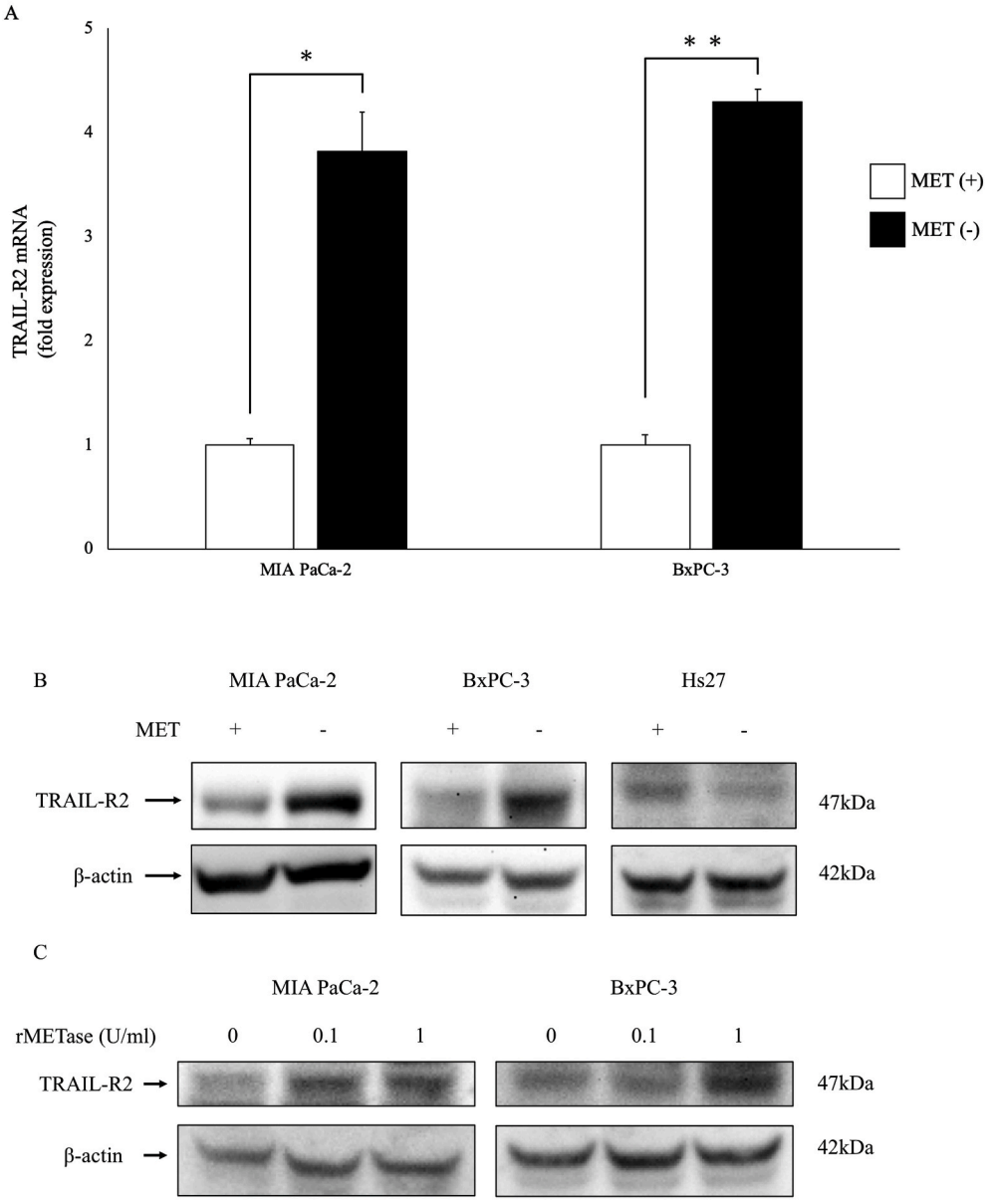

Fig. 3. Methionine restriction induces TRAIL-R2 expression in pancreatic cancer cells in vitro. (A) Cells were cultured in normal medium (MET(+)) or methionine-free medium (MET(−)) for 48 h. TRAIL-R2 mRNA levels were measured by real-time PCR and normalized to expression in pancreatic-cancer cells grown in MET + medium (mean ± SEM, n = 3). (B) Immunoblot of TRAIL-R2 protein expression in pancreatic-cancer cells grown in MET (+) medium or MET(−) medium for 48 h for MIA PaCa-2 or Hs27 cells, and cultured for 24 h for BxPC-3 cells. (C) Immunobloting of TRAIL-R2 protein expression in pancreatic-cancer cells grown in MET (+) medium with or without rMETase for 48 h for MIA PaCa-2 and for 24 h for BxPC-3 cells.

increased in the tumors treated with o-rMETase (Fig. 6A). H & E staining showed that the density of viable cancer cells was decreased in the tumors treated with o-rMETase or tigatuzumab, and the lowest cancer cell density was detected in the tumors treated with the combination of o-rMETase plus tigatuzumab (Fig. 6A). Immuno-histo-chemical (IHC) staining showed that TRAIL-R2 expression was enhanced and MAGED2 expression was suppressed in the o-rMETase-treatment group and combination treatment group.

IHC staining showed that the number of cleaved caspase-3-positive cells was significantly higher in the combination-treatment group than other groups (control; P = 0.003, o-rMETase; P = 0.012, tigatuzumab; P = 0.018) (Fig. 6B). Furthermore, immunoblotting showed that the expression level of TRAIL-R2 was increased in the tumors treated with o-rMETase (Fig. 6C) and that the expression level of procaspase-3 was reduced in the tumors treated with the combination of o-rMETase plus tigatuzumab (Fig. 6D).

These findings suggest that MR using o-rMETase enhanced the antitumor efficacy of tigatuzumab by increasing TRAIL-R2 expression in the pancreatic-cancer orthotopic mouse model.

4. Discussion

In this study, we demonstrated that MR, effected by MET (-) medium, enhanced the efficacy of TRAIL-R2 agonist, tigatuzumab, by increasing TRAIL-R2 expression *in vitro* and also obtained similar results using rMETase *in vivo*. MET dependence is due to the overuse of MET for aberrant trans-methylation reactions in cancer and is possibly the only known general metabolic defect in cancer [5–9]. The overuse of MET by cancer cells for enhanced and unbalanced transmethylation is the basis of the methionine dependence of cancer cells and is termed the “Hoffman effect”, analogous to the Warburg effect of glucose overuse in cancer [14]. PET imaging of cancer with [^{11}C]MET provides a robust

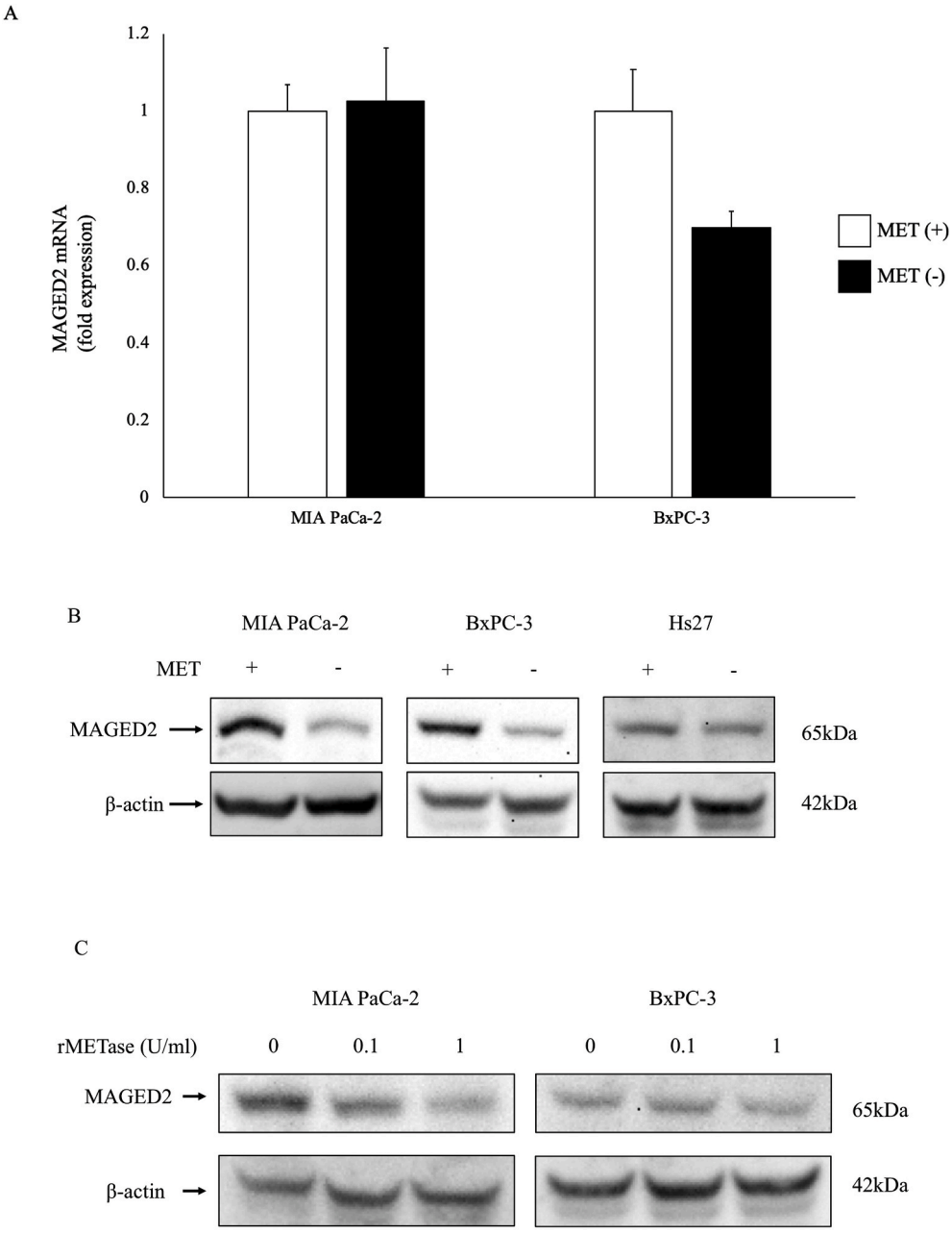

Fig. 4. Methionine restriction reduces MAGED2 protein expression without reducing MAGED2 mRNA expression levels *in vitro*. (A) Cells were cultured in normal medium (MET(+) or methionine-free medium (MET(−) for 48 h. MAGED2 mRNA levels were measured by real-time PCR and normalized to expression in pancreatic cancer cells grown in MET(+) medium (mean ± SEM, n = 3). (B) Immunoblot of MAGED2 protein expression in pancreatic-cancer cells grown in MET(+) medium or MET(−) medium for 48 h for MIA PaCa-2 or Hs27 cells, and cultured for 24 h for BxPC-3 cells. (C) Immunobloting of MAGED2 protein expression in pancreatic-cancer cells grown in MET (+) medium with or without rMETase for 48 h for MIA PaCa-2 and for 24 h for BxPC-3 cells.

signal compared to fluorodeoxyglucose [^{18}F]FDG, suggesting that the Hoffman effect is more prominent compared to the Warburg effect [39] . MR induces cell cycle arrest in S/G_2 phase and apoptosis in cancer cells [10,11]. Therefore, MR enhances the efficacy of cytotoxic chemotherapy drug by S/G_2-phase cell-cycle trap of the cancer cells [7,10,40]. We have previously shown the efficacy of combination treatment with rMETase and cytotoxic agents such as 5-fluorouracil, cisplatinum and gemcitabine [18,41,42]. The results of the present study suggested that rMETase enhances the efficacy of molecular targeting therapy as well by increasing protein expression of a specific receptor. These findings are consistent with the results of previous study with dietary MR and TRAIL-R2 agonist lexatumumab in triple-negative breast cancer [28]. However, the *in vivo* efficacy of MR enabled by rMETase in the present study was much stronger than the previous study in which MR was effected by a low methionine diet [28]. In the previous study using dietary MR, tumor growth was only slower by a TRAIL-R2 agonist [28]. In the present study using o-rMETase to effect MR, the tumor regressed with massive apoptosis when tigatuzumab was combined with o-rMETase. A recent study showed that dietary MR in humans resulted in effects on systemic metabolism that were similar to those obtained in mice [43]. The present results indicate that MR using o-rMETase enhances the efficacy of molecular-targeted therapy as well as cytotoxic agents. rMETase was dosed orally, a much safer route than injection [44].

TRAIL has the ability to induce apoptosis via cross-linking with TRAIL-R1 and TRAIL-R2, expressed by a wide variety of cancer cells, and not normal cells [45]. The specific cancer-targeting capability of TRAIL has attracted great attention worldwide as a potential candidate for cancer therapy. A large number of clinical trials of TRAIL-R2 agonists were performed and the treatments were well tolerated [46–48]. However, the treatment with TRAIL-R2 agonists did not achieve the primary endpoint. Thus, TRAIL-R2 agonists should be used with a treatment that can sensitize the efficacy of TRAIL-R2 agonist. Some drugs were reported to enhance the efficacy of TRAIL-R2 target therapy, such as aspirin, allopurinol and ibuprofen [49–51]. However, these drugs

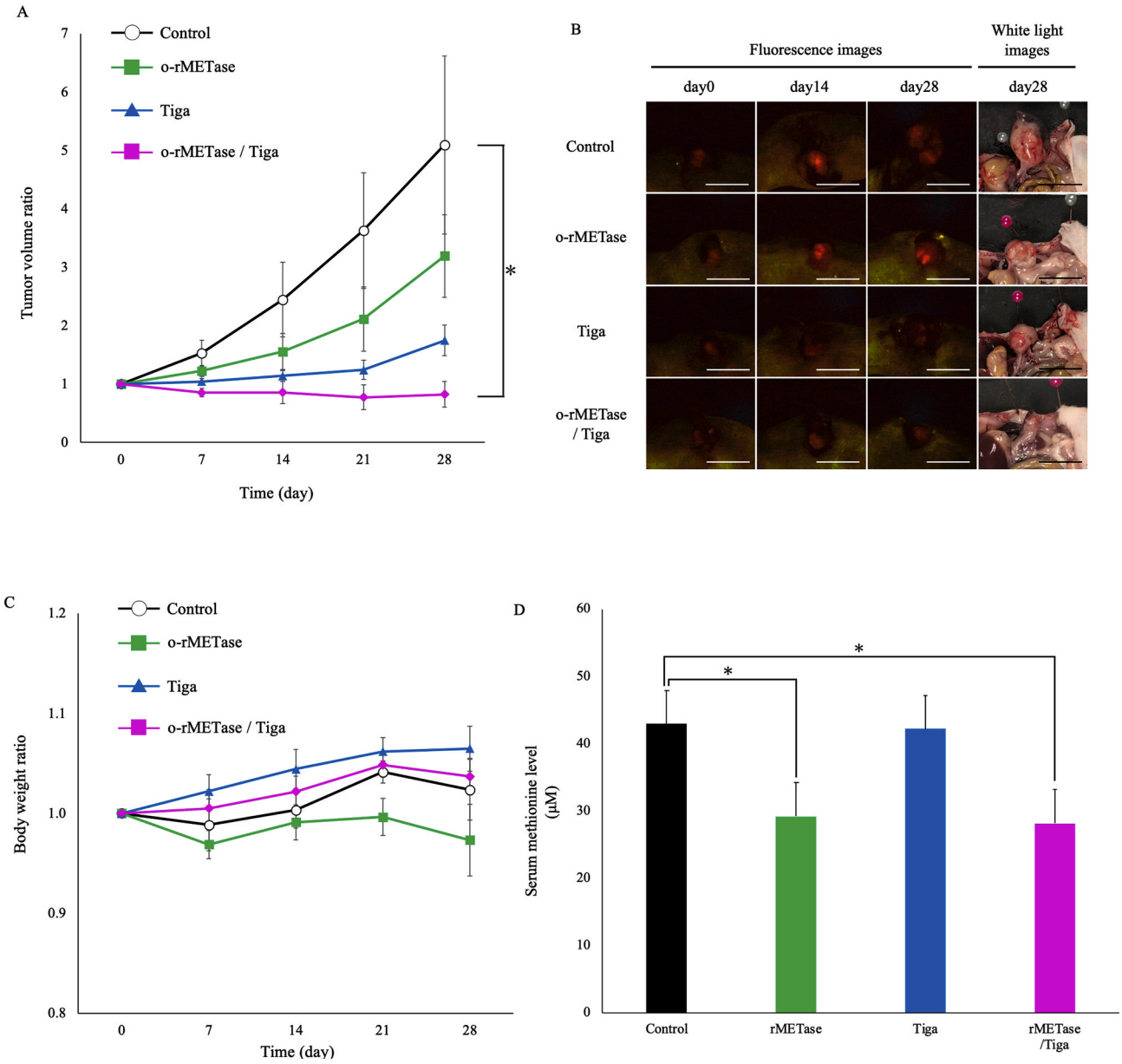

Fig. 5. Oral recombinant methioninase reduces serum methionine levels and enhances the antitumor efficacy of tigatuzumab in a pancreatic cancer orthotopic model. Athymic nu/nu female mice with orthotopic MIA PaCa-2/RFP tumors were randomized four groups (6 mice per group); control, oral recombinant methioninase (o-rMETase, 100U twice a day.), tigatuzumab (3 mg/kg weekly), o-rMETase plus tigatuzumab. (A) Treatment response of the tumor volume ratio in each treatment group (mean ± SEM, n = 6). Black arrow; administration of tigatuzumab. (B) Representative fluorescent and white light images. Scale bar; 20 mm (C) Body weight ratio. (D) Serum methionine levels after treatment (mean ± SEM, n = 5). In (A) and (C), *, $p < 0.05$.

caused adverse events such as bleeding and renal dysfunction. Previous studies showed that both dietary MR and rMETase had no clinical toxicity [43,52]. In addition, in this study, rMETase did not induce apparent side-effects. Therefore, rMETase should be more suitable as enables effective TRAIL-R2 targeted therapy. Treatment of transgenic pancreatic-cancer mouse models will be studied in future experiments. In transgenic mouse models of pancreatic cancer, tumor progression and the microenvironment are more similar to clinical human pancreatic cancer than the xenograft model [53–56]. The pancreatic tumor in the transgenic mouse model has a rich stromal component between the cancer cells and vessels [56]. This characteristic decreases exposure of the tumors to drugs. Efficacy studies of o-rMETase and tigatuzumab on transgenic pancreatic cancer models should be useful to predict the efficacy for human pancreatic cancer.

MAGED2 is a ubiquitously-expressed member of the melanoma-associated antigen (MAGE)-II family that has been implicated in apoptosis-resistance by inhibiting TRAIL-R2 expression and/or antagonizing p53 function [38,57]. Indeed, MAGED2 was previously identified as a negative regulator of TRAIL-R2 expression and TRAIL-induced apoptosis in melanoma cells [38]. Strekalova et al. reported that silencing MAGED2 mimicked the effects of MR, including increased mRNA and expression of TRAIL-R2 and enhanced the efficacy of TRAIL-R2 agonist [28]. We have shown that MR suppresses the MAGED2 protein expression level but did not affect of mRNA level.

In summary, our results suggest that MR enabled the efficacy of TRAIL-R2 targeted therapy of pancreatic cancer by increasing expression of TRAIL-R2 protein. MR, especially o-rMETase, has important clinical potential to overcome this recalcitrant disease, as and much superior than diet-effected MR.

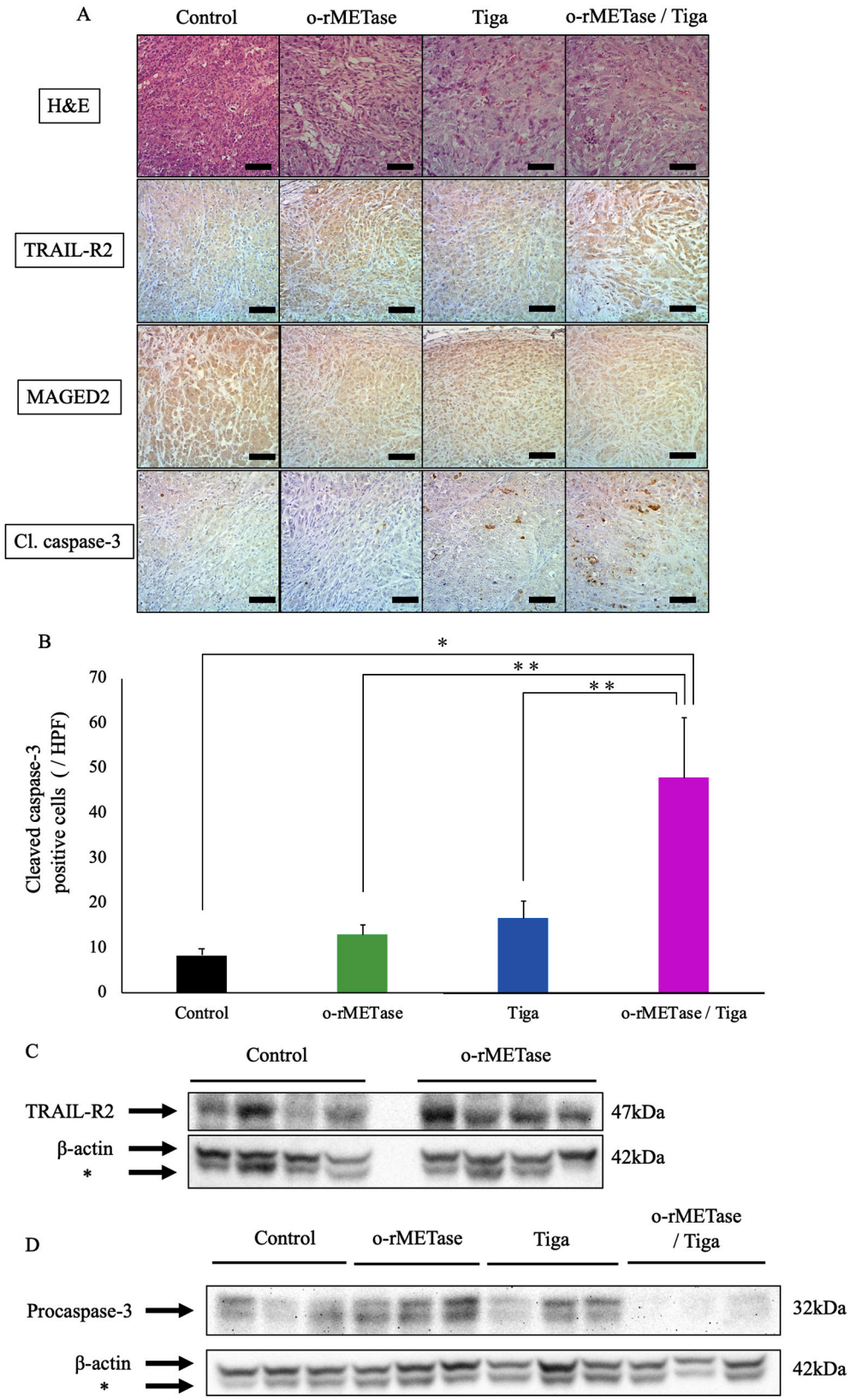

Fig. 6. Oral recombinant methioninase induces TRAIL-R2 expression and tigatuzumab-effected apoptosis in a pancreatic-cancer orthotopic model. (A) Representative images of H & E staining and immunohistochemical staining for TRAIL-R2, MAGED2 and cleaved caspase-3. Scale bar 50 μm. (B) The number of the cleaved caspase-3 positive cells. Five images were randomly obtained in high power fields and the positive cells compared (mean ± SEM, n = 5). *, $p < 0.01$, **, $p < 0.05$. (C) Immunoblot of TRAIL-R2 expression in pancreatic tumors from mice in the control group and the rMETase group (n = 4). *, Non-specific band. (D) Immunoblot of procaspase-3 expression in pancreatic tumors from mice in each group (n = 3). *, Non-specific band.

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Author contributions

JY and RMH were involved in study conception and design. JY, KM, QH, YT, SI, NS, TH, YT, HN, YH, and RM were involved in acquisition of data. JY, KM, QH, YT, SI, NS, TH, YT, HN, YH, RM, SPC, MB, SRS, IE, and RMH analyzed and interpreted the data. JY, RMH and SRS wrote the manuscript. SRS was involved in critical revision of the manuscript. All authors reviewed the manuscript.

Declaration of competing interest

JY, KM, QH, YT, SI, NS, TH, YT, HN and RMH are or were unsalaried associates of AntiCancer Inc. The Authors declare that there are no potential conflicts of interest.

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論文目録

Ⅰ　主論文

Oral recombinant methioninase increases TRAIL receptor-2 expression to regress pancreatic cancer in combination with agonist tigatuzumab in an orthotopic mouse model

Yamamoto J, Miyake K, Han Q, Tan Y, Inubushi S, Sugisawa N, Higuchi T, Tashiro Y, Nishino H, Homma Y, Matsuyama R, Chawla SP, Bouvet M, Singh SR, Endo I and Hoffman RM.

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Ⅱ　副論文

Histone methylation status of H3K4me3 and H3K9me3 under methionine restriction is unstable in methionine-addicted cancer cells, but stable in normal cells

Yamamoto J, Han Q, Inubushi S, Sugisawa N, Hamada K, Nishino H, Miyake K, Kumamoto T, Matsuyama R, Bouvet M, Endo I, and Hoffman RM.

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