Deletion of *MTAP* Highly Sensitizes Osteosarcoma Cells to Methionine Restriction With Recombinant Methioninase

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Abstract. *Background/Aim: Methionine addiction is a fundamental and general hallmark of cancer cells, which require exogenous methionine, despite large amounts of methionine synthesized endogenously. 5-Methylthioadenosine phosphorylase (MTAP) plays a principal role as an enzyme in the methionine-salvage pathway, which produces methionine and adenine from methylthioadenosine and is deleted in 27.5% to 37.5% of osteosarcoma patients. Materials and Methods: Human osteosarcoma cell lines U2OS, SaOS2, MNNG/HOS (HOS) and 143B, were used. The MTAP gene was knocked out in U2OS with CRISPR/Cas9. 143B and HOS have an MTAP deletion and SaOS2 is positive for MTAP. MTAP was determined by western blotting. The four cell lines were compared for sensitivity to recombinant methioninase (rMETase). Results: MTAP-deleted osteosarcoma cell lines MNNG/HOS and 143B were significantly more sensitive to rMETase than MTAP-positive osteosarcoma cell lines U2OS and SaOS2. In addition, MTAP knock-out U2OS cells were more sensitive to rMETase than the parental MTAP-positive U2OS cells. Conclusion: The present results demonstrated that the absence of MTAP sensitizes osteosarcoma cells to methionine restriction by rMETase, a promising clinical strategy.*

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Methionine addiction, discovered by one of us (RMH) in 1976 (1), is a general and fundamental hallmark of cancer cells and is termed the Hoffman effect. Methionine addiction occurs because cancer cells have highly overactive transmethylation reactions (1-5).

Cancer cells under methionine restriction arrest in S/G_2 phase in contrast to normal cells, which arrest in G_0 phase (6).

5-Methylthioadenosine phosphorylase (MTAP) is a rate-limiting enzyme in the methionine salvage pathway. This pathway produces methionine and adenine from 5'-methylthioadenosine (MTA) (7, 8). Between 27.5% to 37.5% of patients with osteosarcoma have a homozygous *MTAP* deletion (9, 10).

Cancer cells with MTAP deletion are sensitive to methionine restriction (11-19) but have not been tested with recombinant methioninase (rMETase), the most efficient means of methionine restriction, which is the topic of the present report.

Materials and Methods

Cell culture. U2OS, SaOS2, MNNG/HOS (HOS), 143B human osteosarcoma cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (10-013-CV; Corning, Corning, NY, USA) with 10% fetal bovine serum (FBS) and 1 IU/ml penicillin/streptomycin.

Recombinant methioninase. rMETase is a homotetrameric enzyme, with a 172-kDa molecular mass. Production of rMETase was as previously reported, using fermentation, a heat step, polyethylene-glycol precipitation and gel filtration (20).

MTAP gene-knockout. U2OS cells were transfected using CRISPR/Cas9 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to the protocol provided by the company. Briefly, cells were cultured in six-well plates (2.5×10^5 cells/well) in DMEM (1 ml/well) and incubated overnight. After cells grew to approximately 60% confluence, DMEM was aspirated and DMEM

containing 10% FBS, but no antibiotics, were added and cells were incubated for 1 h. *MTAP* was knocked out in U2OS cells using CRISPR/Cas9 with the following materials and methods: MTAP Double Nickase plasmid (2 μg/20 μl) (sc-406223-NIC; Santa Cruz Biotechnology) or Control Double Nickase Plasmid (2 μg/20 μl) (sc-437281; Santa Cruz Biotechnology) were added to 130 μl Plasmid Transfection Medium (sc-108062; Santa Cruz Biotechnology), bringing the final volume to 150 μl (solution A). UltraCruz Transfection Reagent (sc-395739, 10 μl; Santa Cruz Biotechnology) was added to 140 μl Plasmid Transfection Medium (sc-108062; Santa Cruz Biotechnology), bringing the final volume to 150 μl (solution B). Solution A was added dropwise to solution B, bringing the total volume to 300 μl, immediately vortexed, and incubated at room temperature for 20 min (solution C). DMEM, which contains 10% FBS but no antibiotics, was aspirated from the wells and a total of 300 μl of solution C was added to the wells and the plates were incubated for 72 h at 37°C with 5% CO_2. After incubation, solution C was aspirated, 2 ml of DMEM containing 10% FBS and 5 μg/ml puromycin dihydrochloride (sc-108071; Santa Cruz Biotechnology) was added and plates were then incubated for 7 days at 37°C with 5% CO_2, at which time *MTAP* gene knocked-out cells were selected by resistance to puromycin (see below). During the incubation, the medium was changed once.

Limiting-dilution cloning of MTAP gene-knockout cells. After cell selection with puromycin for 7 days, the puromycin-containing medium was aspirated, and cells were cultured with DMEM containing 10% FBS and 1 IU/ml penicillin/streptomycin. At the end of the 10-day culture period, cells were harvested with trypsin and suspended at a density of 10 cells/ml in DMEM. The cells were then added to 96-well plates (1 cell/well, 100 μl) and incubated at 37°C with 5% CO_2. Wells containing a single-cell colony were marked and were allowed to incubate for 2 more weeks to expand the colony. The individual colonies were then cultured in T25 cell-culture flasks for 7 days. The cells were harvested with trypsin and proteins were extracted. Immunoblotting for MTAP was performed to confirm complete knock-out of the *MTAP* gene.

Immunoblotting. Cells with and without the *MTAP* gene were cultured in T25 cell-culture flasks. They were then lysed, and protein was extracted using RIPA Lysis and Extraction buffer (Thermo Fisher Scientific, Waltham, MA, USA) with 1% Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific). Protein extract samples were loaded onto 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and transferred to 0.45 μm polyvinylidene difluoride membranes (GE10600023; GE Healthcare, Chicago, IL, USA) after electrophoresis. The membranes were blocked using Bullet Blocking One for Western Blotting (Nakalai Tesque Inc., Kyoto, Japan). Antibody to MTAP (ab126770, 1:10,000; Abcam, Cambridge, UK) and anti-β actin (20536-1-AP, 1:1,500; Proteintech, Rosemont, IL, USA) were used. β-Actin was used as a loading control. As a second antibody, horseradish-peroxidase-conjugated anti-rabbit IgG (SA00001-2, 1:20,000; Proteintech, Rosemont, IL, USA) was used. The signals were detected with a UVP ChemStudio (Analytik Jena, Upland, CA, USA) using a Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA) to qualitatively visualize immunoreactivity.

rMETase sensitivity assay. Sensitivity to rMETase of osteosarcoma cell lines was assessed using the WST-8 reagent (Dojindo
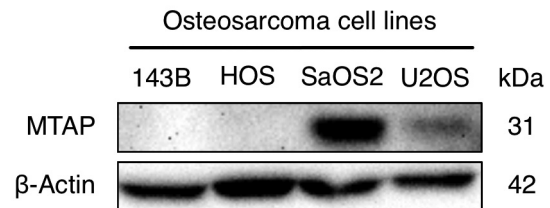

Figure 1. *Immunoblotting of 5-methylthioadenosine phosphorylase (MTAP) and β-actin in osteosarcoma cell lines U2OS, SaOS2, HOS and 143B.*

Laboratory, Kumamoto, Japan). The cells were cultured in 96-well plates at the following densities for each cell line: U2OS: 1.5×10^3 cells/well; SaOS2: 2.5×10^3 cells/well; HOS: 1.0×10^3 cells/well; and 143B: 7.5×10^2 cells/well; in DMEM (100 μl/well) and incubated overnight at 37°C with 5% CO_2. Cells were treated with rMETase at different concentrations, between 0.25 U/ml and 4.0 U/ml, for 96 h at 37°C with 5% CO_2. After rMETase treatment, 10 μl WST-8 solution was added to each well and the cells were incubated for an additional 1 h under the same conditions. Absorbance was then measured with a microplate reader (Sunrise; Tecan, Männedorf, Switzerland), at a wavelength of 450 nm. Drug-sensitivity curves were constructed using Microsoft Excel for Mac 2016 ver. 15.52 (Microsoft, Redmond, WA, USA) and half-maximal inhibitory-concentration (IC_{50}) values were calculated with ImageJ ver. 1.53k (National Institutes of Health, Bethesda, MD, USA). Experiments were repeated twice for each cell line, each in triplicate.

Statistical analysis. All statistical analyses were performed with JMP pro ver. 15.0.0 (SAS Institute, Cary, NC, USA). Tukey-Kramer analysis was performed to compare each group separately for a significant difference. The Dunnett test was performed to compare each of the means with the control. Bar graphs show the mean and error bars show standard deviation of the mean. A probability value of ≤0.05 was defined as statistically significant.

Results

The *MTAP gene is expressed in U2OS and SaOS2 but not in HOS and 143B osteosarcoma cell lines.* *MTAP* gene expression was evaluated by immunoblotting in osteosarcoma cell lines. *MTAP* expression in U2OS and SaOS2, but not in HOS and 143B observed in the present report, is consistent with previous reports (9, 10) (Figure 1).

Osteosarcoma cell lines with an *MTAP deletion are more sensitive to rMETase than MTAP-positive cells.* rMETase inhibited the proliferation of *MTAP*-positive osteosarcoma cell lines with the following IC_{50} values: U2OS: 0.74 U/ml and SaOS2: 0.72 U/ml. In contrast, rMETase inhibited *MTAP*-negative cells with the following IC_{50} values: HOS: 0.22 U/ml and 143B: 0.24 U/ml, demonstrating that *MTAP*-negative cancer cells were much more sensitive to rMETase than *MTAP*-positive cells ($p<0.001$) (Figure 2).
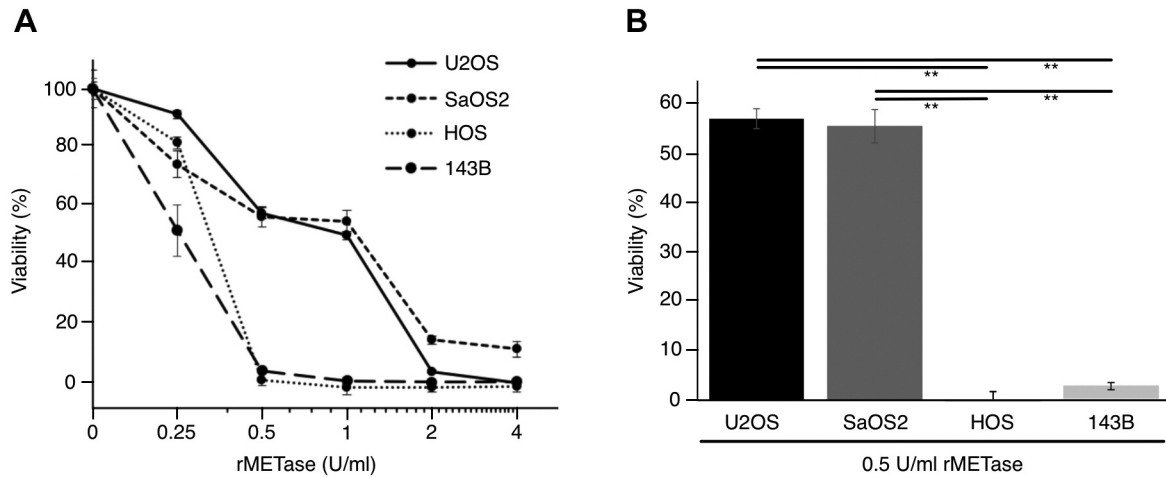
Figure 2. *A: Sensitivity of human osteosarcoma cell line U2OS, SaOS2, HOS and 143B to treatment with various concentrations of recombinant methioninase (rMETase) (mean±SD, n=3). B: Comparison of the efficacy of rMETase (0.5 U/ml) on the osteosarcoma cell lines. **Significantly different at p<0.001.*
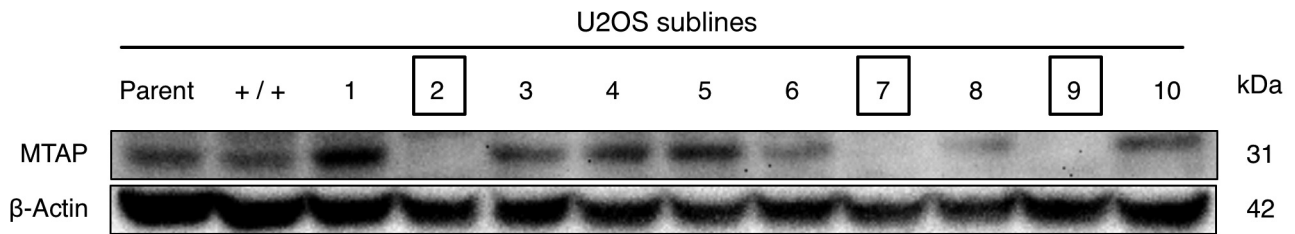

Figure 3. *Immunoblotting of 5-methylthioadenosine phosphorylase (MTAP) and β-actin in parent, control, and MTAP knock-out U2OS cell lines. U2OS sublines numerated in squares have MTAP knocked out.*

MTAP knock-out U2OS cells are more sensitive to rMETase than parental MTAP-positive cells. The *MTAP* gene was knocked out in U2OS cells (Figure 3). Three new independent *MTAP*-knockout sublines were designated as U2OS *MTAP*$^{-/-}$#2; U2OS *MTAP*$^{-/-}$#7; and U2OS *MTAP*$^{-/-}$#9. U2OS *MTAP*$^{+/+}$, which was treated with a control plasmid, was therefore used as the control (Figure 3). Compared to the U2OS *MTAP*$^{+/+}$ control, all three *MTAP* knock-out sublines were more sensitive to rMETase, with the following IC$_{50}$ values: U2OS *MTAP*$^{+/+}$ control: 1.14 U/ml; U2OS *MTAP*$^{-/-}$#2: 0.31 U/ml; U2OS *MTAP*$^{-/-}$#7: 0.47 U/ml; and U2OS *MTAP*$^{-/-}$#9: 0.45 U/ml. U2OS *MTAP*$^{-/-}$#2 (*p*<0.001), U2OS *MTAP*$^{-/-}$#7 (*p*=0.001), U2OS *MTAP*$^{-/-}$#9 (*p*=0.0013) cells were much more sensitive to rMETase at 0.5 U/ml than the U2OS *MTAP*$^{+/+}$ control (Figure 4).

Discussion

MTAP is the rate-limiting enzyme in the methionine-salvage pathway and has global effects on cellular methylation due

to the utilization of *S*-adenosyl methionine in the methionine salvage pathway (11-19). In addition, the *MTAP* gene is adjacent to the tumor suppressor P16 (13, 19) and often both are deleted together.

The present study showed osteosarcoma cell lines with *MTAP* deletion to be significantly more sensitive to rMETase than *MTAP*-positive ones. *MTAP*-knock-out U2OS cells were more sensitive to rMETase than parental MTAP-positive U2OS cells, which supports the concept that *MTAP* deletion sensitizes osteosarcoma cell lines to rMETase. The present study is the first to show that the absence of MTAP highly sensitizes cancer cells to rMETase. This was shown by direct comparison of sensitivity to rMETase of an MTAP-positive cell line with its sublines in which *MTAP* was knocked out; the MTAP knockout sublines of U2OS cells were significantly more sensitive to rMETase than the parental MTAP-positive cells. In addition, when the MTAP-positive and MTAP-negative cell lines were compared, it was shown that the MTAP-negative cell lines, HOS and 143B, were more sensitive to rMETase.
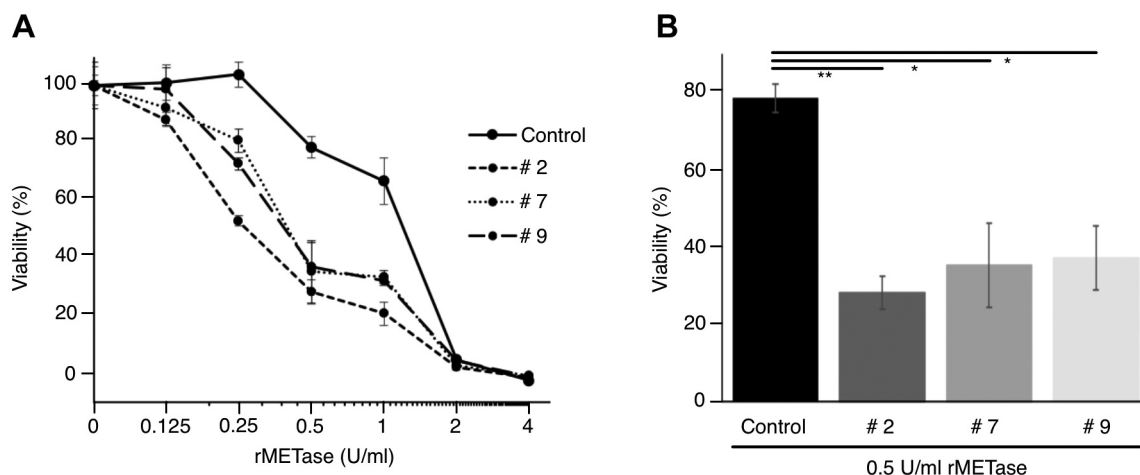
Figure 4. *A: Sensitivity to recombinant methioninase (rMETase) of 5-methylthioadenosine phosphorylase (MTAP)$^{+/+}$ control and MTAP knock-out U2OS sublines #2, #7 and #9 (mean±SD, n=3). B: Comparison of the efficacy of 0.5 U/ml rMETase against MTAP$^{+/+}$ control cells and MTAP$^{-/-}$ sublines #2, #7 and #9. Significantly different at *p<0.01 and **p<0.001.*

A previous study by Tang *et al.* suggested that MTAP is related to methionine dependence (addiction) in that MTAP-negative cells were unable to proliferate when homocysteine replaced methionine in the culture medium, while MTAP-positive cancer cells were still able to proliferate (12). However, their studies were defective since the FBS used in the medium was not dialyzed, and thereby actually contributed significant amounts of methionine to the 'methionine-free' culture medium. It was previously shown that even a very small amount of methionine (1 μM) highly stimulated cancer-cell proliferation in culture medium containing homocysteine instead of methionine (1). Previous studies also showed that MTAP replacement in MTAP-negative cancer cells did not revert methionine dependence to methionine independenc(12). Although the study of Tang *et al.* (12) is an old one, it demonstrates that care must be taken to ensure that medium termed 'methionine-free' is indeed free of methionine. The present study demonstrates that it is much more straight forward to determine methionine addiction with rMETase. Methionine dependence (addiction) is due to excessive transmethylation in cancer cells and is independent of MTAP (1-5).

Future studies will focus on potential therapy for patients with tumors with *MTAP* deletions with rMETase, using patient-derived orthotopic xenograft mouse models of osteosarcoma, which we have established to identify potential effective treatment strategies in our laboratory (21-23).

MTAP-deficient cancer cells are also sensitive to purine inhibitors, such as 6-mercaptopurine, methotrexate, pemetrexed, azaserine, azathioprine, L-alanosine and mizoribine (11, 24-28). Future studies will evaluate the efficacy of rMETase in combination with inhibitors of purine synthesis, in both MTAP-positive cancer and MTAP-negative cancer, which may be more

malignant (29) possibly due to co-deletion of *P16* with the *MTAP* gene (19). Methionine addiction is a fundamental (1-5, 30-37) and general (35-37) hallmark of cancer which is enhanced as a therapeutic target (38) by MTAP deletion.

Conflicts of Interest

Authors' Contributions

YA, YT and RMH were involved in study conception and design. YA, JY, KH, NM and YK were involved in acquisition of data. YA, YT, JY, KH, NM, YK and RMH analyzed and interpreted data. YA, YT and RMH wrote the article. All Authors reviewed and approved the article.

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