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Oral recombinant methioninase combined with oxaliplatinum and 5-fluorouracil regressed a colon cancer growing on the peritoneal surface in a patient-derived orthotopic xenograft mouse model

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ABSTRACT

The aim of this study was to determine the efficacy of oral recombinant methioninase (o-rMETase) on a model of colon cancer growing on the peritoneal surface using a patients-derived orthotopic xenograft (PDOX) nude mouse model. Forty PDOX mouse models with colon cancer growing on the peritoneum were divided into 4 groups of 10 mice each by measuring the tumor size and fluorescence intensity: untreated control; 5-fluorouracil (5-FU) (50 mg/kg, once a week for two weeks, ip) and oxaliplatinum (OXA) (6 mg/kg, once a week for two weeks, ip); o-rMETase (100 units/day, oral 14 consecutive days); combination 5-FU + OXA and o-rMETase. All treatments inhibited tumor growth compared to the untreated control. The combination of 5-FU + OXA plus o-rMETase was significantly more efficacious than the control and each drug alone and was the only treatment that caused tumor regression. The present study is the first demonstrating the efficacy of o-rMETase combination therapy on a PDOX model of peritoneal colon cancer, suggesting potential clinical development of o-rMETase in a recalcitrant cancer.

1. Introduction

The elevated methionine (MET) requirement of cancer cells is referred to as MET dependence (Hoffman, 1984, 2015a, 2017; Stern and Hoffman, 1984; Wang et al., 2019). The elevated MET use in cancer is called the "Hoffman effect" analogous to the Warburg effect of excess glucose consumption by cancers. Comparison of radioactive MET- and glucose- PET (positron-emission tomography) imaging has shown a stronger signal with MET suggesting that the Hoffman effect is more pronounced than the Warburg effect (Hoffman, 2015a, 2017; Xu et al., 2017).

MET restriction by recombinant methioninase (rMETase) can inhibit the growth of cancer cells in vitro and in vivo (Hoffman, 2015a, 2017). rMETase has been used as a treatment strategy for various types of cancer (Hoffman, 2015a; Kreis and Hession, 1973; Tan et al., 2010; Xin et al., 2013, 2018; Yano et al., 2014, 2016).

Previous studies have shown that intra-peritoneal recombinant methioninase injection (ip-rMETase) was effective against patient-derived orthotopic xenograft (PDOX) mouse models of recalcitrant cancer (Kawaguchi et al., 2017a,2018a,c; Igarashi et al., 2018a,b]. Recently, we reported that oral recombinant methioninase (o-rMETase) was significantly more effective than intraperitoneal injection rMETase (ip-rMETase) indicating the potential widespread use of rMETase for cancer treatment (Higuchi et al., 2018; Higuchi et al., 2019; Kawaguchi et al., 2018a; Kawaguchi et al., 2017b; Kawaguchi et al., 2018d; Oshiro et al., 2019; Park et al., 2019a).

Orthotopic implantation of intact tumor tissue in appropriate mouse models can result in metastasis resembling the clinical pattern, unlike subcutaneous transplantation. Our laboratory has used surgical orthotopic implantation (SOI) to establish PDOX nude-mouse models from patient tumor specimens (Fu and Hoffman, 1993; Hiroshima et al., 2014; Hiroshima et al., 2015; Hoffman, 2015b; Igarashi et al.,

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2018a,2018b,c; Kawaguchi et al., 2017a,2017b; Kawaguchi et al., 2018c,d,e; Kawaguchi et al., 2016; Kiyuna et al., 2016; Metildi et al., 2014; Murakami et al., 2016; Wang et al., 1992; Yamamoto et al., 2016). The PDOX orthotopic models are much more patient-like than the ectopic subcutaneous models (Hoffman, 2015b). However, in orthotopic models, it is difficult to visualize tumor growth. To address this problem of imaging such orthotopic tumor grafts, we have recently developed the technology to introduce fluorescent protein-expressing stroma into tumors by passaging tumor grafts through transgenic nude mice expressing fluorescent protein (Suetsugu et al., 2012).

The present report demonstrates the efficacy of o-rMETase using a PDOX model of colon cancer growing on the peritoneal surface in nude mouse, with brightly labeled red fluorescent protein (RFP)-expressing stroma for imaging.

2. Materials and methods

2.1. Mice

Athymic nu/nu nude mice and transgenic RFP expressing athymic nu/nu mice (four to 6 weeks) were obtained from AntiCancer Inc. (San Diego, CA). All surgical procedures and imaging were performed in accordance with an AntiCancer Institutional Animal Care and Use Committee (IACUC)-protocol specifically 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. Mouse housing, feeding, surgical process, and imaging were conducted, and mice were humanely sacrificed as previously described (Miyake et al., 2019).

2.2. Patient-derived tumor

The primary tumor was resected from a patient diagnosed with colon cancer. The stage of primary colon cancer was T4aN1bM0. Genetic analysis of the tumor will be done in future experiments. After 8 months, peritoneal recurrence occurred and the patient was treated with surgical resection and chemotherapy at the Division of Surgical Oncology, University of California, San Diego (UCSD). A fresh sample of the colon cancer was obtained immediately after patient surgery with informed patient consent and IRB (institutional review board) approval. The tumor was cut into fragments and initially implanted subcutaneously in nude mice. The subsequent subcutaneous tumor was harvested and used for orthotopic implantation.

2.3. Establishment of a PDOX model of colon cancer with red fluorescence

Patient colon cancer growing in nude mice was harvested, cut into 5 mm fragments, and implanted subcutaneously in transgenic RFP-expressing nude mice. After two passages, tumors stably containing RFP-expressing stromal cells were obtained and cut into fragments (Fig. 1). After non-transgenic nude mice were anesthetized with ketamine, a 1–2 cm skin incision was made at the midline of the abdomen. Surgical sutures (8-0 nylon) were used to implant tumor fragments onto the peritoneum. Wounds were closed using 6-0 nylon sutures.

2.4. Production of rMETase

Recombinant L-methionine-α-deamino-γ-mercaptomethane lyase (recombinant methioninase [rMETase]) [EC 4.4.1.11] from *Pseudomonas putida* has been previously cloned and was produced in *Escherichia coli* (AntiCancer, Inc., San Diego, CA) (Kawaguchi et al., 2018c). rMETase is a homotetrameric PLP enzyme of 172-kDa molecular mass (Hoffman, 2015a).

2.5. Treatment study design in the PDOX model of colon cancer growing on the peritoneal surface

Four weeks after surgical orthotopic implantation of RFP-expressing colon cancer to non-transgenic nude mice, non-invasive external red fluorescence imaging was performed on all mice, which were divided into 4 groups by measuring the tumor size and fluorescence intensity.

The first group served as a negative control and did not receive treatment. Mice in the second group were treated once a week for two weeks with intraperitoneal injection of 50 mg/kg 5-FU, and 6 mg/kg OXA. Mice in the third group received 100 units/day of o-rMETase by gavage for 2 weeks. Mice in the fourth group received the combination of all 3 drugs.

2.6. Fluorescence imaging of peritoneal tumors and measurement of tumor weight and volume

Four weeks after implantation, mice were anesthetized for measurement of tumor size by non-invasive external red fluorescence imaging with the UVP ibox (Analytik Jena, Germany). External red fluorescence images were obtained twice a week. Fluorescence intensity was measured and calculated using the UVP ibox software. Six weeks after RFP-expressing tumors were implanted, mice were then sacrificed for direct measurements of tumor weight and volume. Frozen tissue sections were observed for fluorescence with an FV1000 confocal laser microscope (Olympus Corp, Tokyo Japan). Excitation wavelength for RFP fluorescence was 559 nm. Tissues were viewed under 4x and 20x objective lenses.

2.7. Statistical analysis

Differences in the weight and volume of the tumors between the groups were assessed for significance using the student's t test. A p-value of < 0.05 was considered statistically significant.

3. Results

3.1. Non-invasive red fluorescence imaging of colon cancer growing on the peritoneal surface of the PDOX model

Orthotopically-implanted tumors grew in the peritoneum of non-transgenic nude mice. These tumors had sufficient red fluorescent stroma from previous growth in RFP nude mice to permit non-invasive imaging through the abdominal wall (Fig. 1A-D). Confocal laser microscopy showed persistence of the RFP-expressing stroma in frozen sections (Fig. 2A, B).

3.2. Tumor histology

Histologically, the untreated control tumor mainly comprised viable carcinoma cells. (Fig. 2C) In contrast, tumor treated with the combination of 5-FU, OXA and rMETase showed reduction of cancer cells as well as necrosis (Fig. 2D).

3.3. Treatment efficacy

At 6 weeks after implantation and completion of treatment, tumor weight was as follows; un-treated control: 136.6 ± 35.1 mg; 5-FU + OXA: 73.9 ± 32.3 mg; o-rMETase: 101.8 ± 24.6 mg; combination of 5-FU + OXA with o-rMETase: 50.5 ± 9.7 mg. 5-FU + OXA combined with o-rMETase was significantly more effective than both 5-FU + OXA alone ($p = 0.041$) and o-rMETase alone ($p = 0.023$) (Fig. 3A). Tumor volume was as follows; untreated control: 54.5 ± 13.1 mm^3; 5-FU + OXA: 27.7 ± 8.9 mm^3; o-rMETase: 33.7 ± 6.7 mm^3; combination of 5-FU + OXA with o-rMETase: 23.8 ± 6.5 mm^3 (Fig. 3B). All treatments inhibited tumor growth

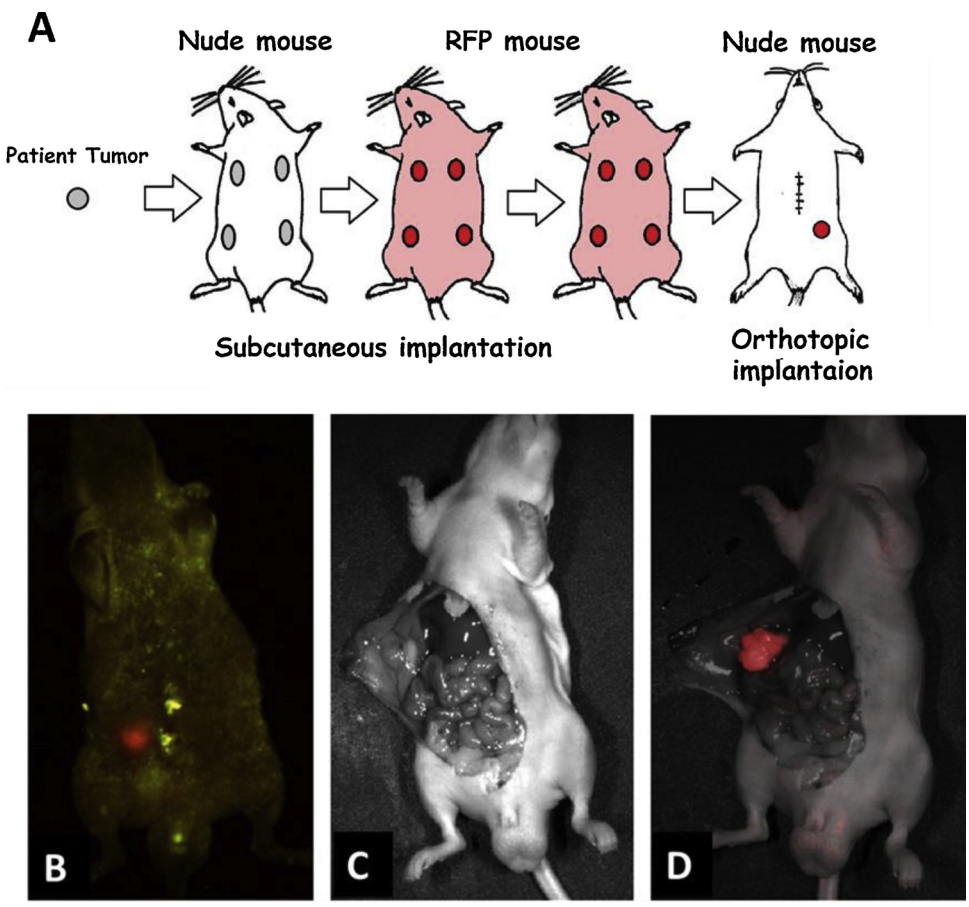

Fig. 1. (A) Experimental schema used to develop imageable PDOX model of human colon tumor growing on the peritoneal surface. (B) Non-invasive red fluorescence image of colon cancer growing on the peritoneal surface, (C) Bright field image after laparotomy. (D) Red fluorescent image after laparotomy (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

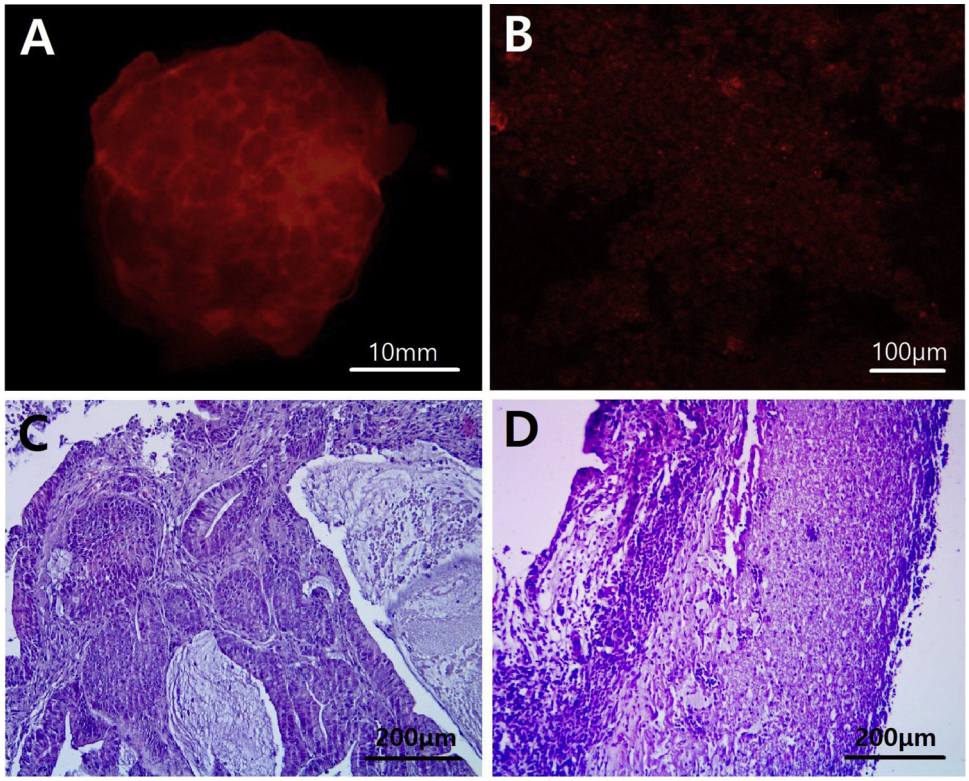

Fig. 2. Confocal laser microscope image of colon cancer growing on the peritoneal surface in frozen section, (A) × 10, (B) × 60. (C, D) Tumor histology (C) untreated control (D) combination treatment with 5-FU + OXA and o-rMETase.

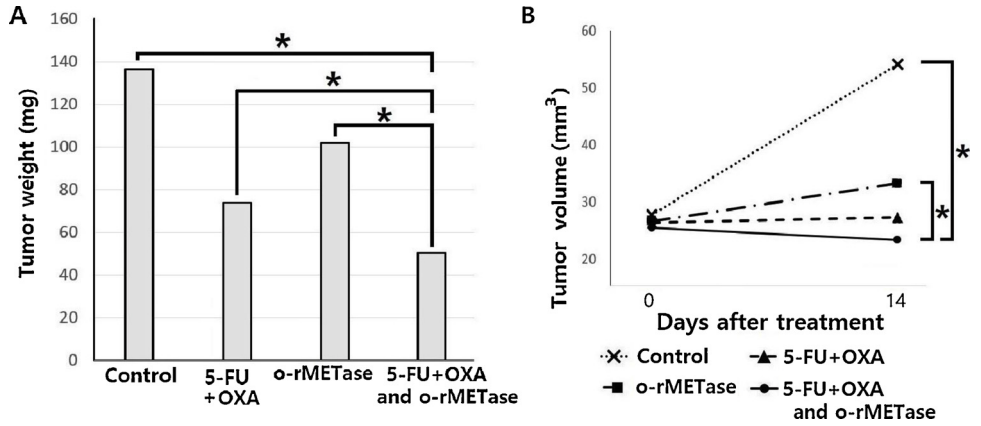

Fig. 3. Treatment efficacy of 5-FU + OXA, o-rMETase and their combination in the colon cancer growing on the peritoneal surface in a PDOX model (A) Tumor weight of treatment groups, (B) Tumor volume of treatment groups. * $p < 0.05$.

compared to the untreated control group. 5-FU + OXA combined with o-rMETase was significantly more effective than o-rMETase alone ($p < 0.01$). Tumor volume of the combination 5-FU + OXA and o-rMETase was smaller than the volume of 5-FU + OXA group, but it was not statistically significant ($p = 0.67$) (Fig. 3B). Only the combination of o-rMETase with 5-FU + OXA regressed the colon cancer growing on the peritoneal surface in the PDOX model.

Using non-invasive external fluorescence imaging, serial fluorescence intensity was calculated. The fluorescence intensity of the combination of o-rMETase with 5-FU + OXA was statistically-significantly lower than the other groups ($p = 0.025$) (Fig. 4A). The fluorescence intensity of the combination of o-rMETase with 5-FU + OXA was lower than 5-FU + OXA group, but it was no significantly significant ($p = 0.068$).

3.4. Body weight

Body weight loss was observed in the 5-FU and OXA groups only. rMETase alone and the untreated control group did not have statistically significant body weight loss (Fig. 4B).

4. Discussion

Recently, a paper appeared with the title “The new anticancer era: tumor metabolism targeting” (Borriello and Della Ragione, 2017). However, this “new anticancer era” started in 1959 with the observation of Sugimura et al. that depriving animals of MET arrested tumor growth (Sugimura et al., 1959). The Warburg effect refers to the significantly increased uptaked glucose by cancer cells. In addition to calorie restriction, specific amino acid restriction has been studied in the past to treat cancer (Lien and Vander Heiden, 2019). Cancer cells are more MET-dependent than normal cells, and simple dietary MET restriction has been shown to reduce the proliferation of numerous cancer cell lines (Tan et al., 2010). Furthermore, MET restriction is known to extend the life-span of various rat strains, indicating that basic health is not threatened by MET restriction (Orentreich et al., 1993; Zimmerman et al., 2003).

Our laboratory has developed PDOX model of cancer for discovery of transformative therapy as well as for individualized therapy. O-rMETase could inhibit tumor growth in PDOX nude mouse models of various types of cancer (Higuchi et al., 2018; Higuchi et al., 2019; Kawaguchi et al., 2018a,2018b; Miyake et al., 2018; Oshiro et al., 2019; Park et al., 2019a). However, the present study is the first to show o-rMETase inhibits colon cancer growing on the peritoneal surface. We not only showed that the efficacy of fluorescence imaging using mouse model of malignant tumors but also correlation between the tumor fluorescence area/intensity and tumor weight/volume (Miwa et al., 2014a,b, 2016; Park et al., 2019a,b). The progression of the colon cancer peritoneal growing could be determined serially by non-invasive external fluorescence imaging with the iBox as described (Miwa et al., 2014a,b, 2016; Park et al., 2019a,b) due to labeling of tumor stroma with RFP.

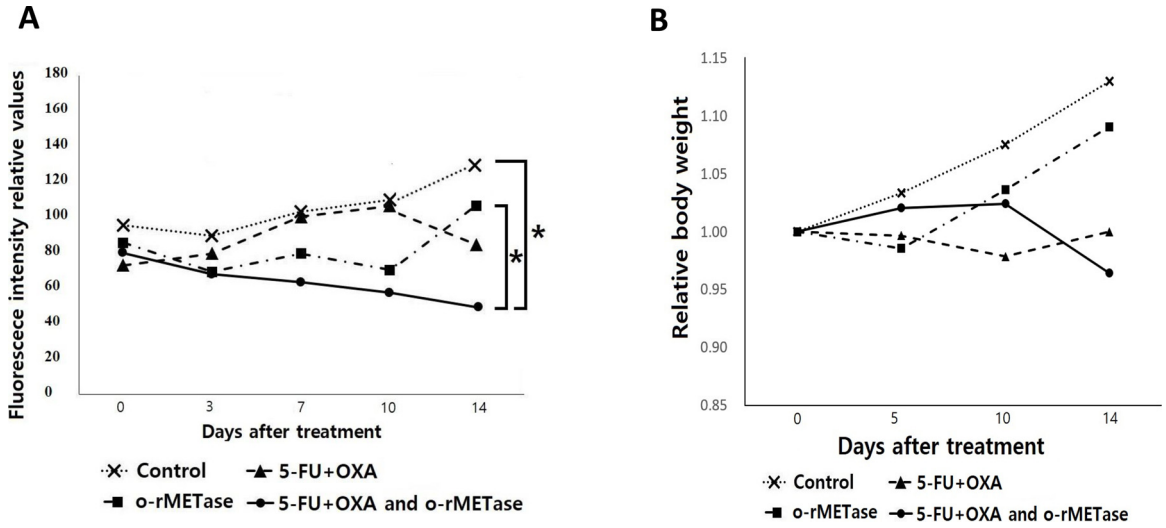

Fig. 4. (A) Time-course fluorescence intensity of the treatment groups. (B) Effect of 5-FU + OXA, rMETase and their combination on mouse body weight.

rMETase administered orally has little side effects. The present study suggests that o-rMETase used in combination with 5-FU and OXA could regress the peritoneally-growing colon cancer in the PDOX model. rMETase has promise as a novel cancer therapeutic in combination with cisplatinum for human colon cancer. Future studies will test this and other combinations with o-rMETase against additional important tumor types.

Declaration of Competing Interest

JHP, HO, KM, TH, SNY, ZZ, NS, JY and RMH are or were unsalaried associates of AntiCancer Inc. QH, MZ and YT are employee of AntiCancer Inc. AntiCancer Inc. uses PDOX models for contract research. The Authors declare that there are no potential conflicts of interest.

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