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Oral Methioninase Inhibits Recurrence in a PDOX Mouse Model of Aggressive Triple-negative Breast Cancer

HYE IN LIM[1,2,3], KAZUYUKI HAMADA[1,2], JUN YAMAMOTO[1,2], QINHONG HAN[1], YUYING TAN[1], HEE JUN CHOI[3], SEOK JIN NAM[4], MICHAEL BOUVET[2] and ROBERT M. HOFFMAN[1,2]

[1]*AntiCancer Inc, San Diego, CA, U.S.A.;*
[2]*Department of Surgery, University of California, San Diego, CA, U.S.A.;*
[3]*Department of Surgery, Samsung Changwon Hospital, Sungkyunkwan University School of Medicine, Changwon, Republic of Korea;*
[4]*Division of Breast Surgery, Department of Surgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Republic of Korea*

Abstract. *Background/Aim: The aim of the study was to use a triple-negative breast cancer (TNBC) patient-derived orthotopic xenograft (PDOX) model to examine the efficacy of oral recombinant methioninase (o-rMETase) against this recalcitrant disease. Materials and Methods: The TNBC tumor from a patient was implanted in the right 4th inguinal mammary fat pad of nude mice. Two weeks later, the mice underwent tumorectomy with grossly-negative surgical margins. Two days after tumorectomy the mice were divided in two groups: one control and one treated with o-rMETase. Results: Tumors recurred in all mice. On day 11, the mean recurrent tumor volumes were 936.7 mm^3 in the control group and 450.9 mm^3 in the o-rMETase group (p<0.05). On day 15, the mean recurrent tumor volumes were 3392.5 mm^3 in the control group and 1603.5 mm^3 in the o-rMETase group. The mean recurrent tumor weights were 2.1 g in the control group and 1.1 g in the o-rMETase group on day 15. Conclusion: o-rMETase is an effective adjuvant treatment for aggressive TNBC.*

Breast cancer is the most common cause of cancer-related death among females worldwide (1). Between 15~20% of breast cancer patients are diagnosed with triple-negative breast cancer (TNBC) based on the absence of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER-2) (2). Unlike other types of breast cancer, TNBC has a poor prognosis with first-line chemotherapy such as anthracyclines and taxanes, and does not have additional treatment options, such as hormonal treatments or targeted therapy, following conventional first-line adjuvant chemotherapy. For this reason, novel therapeutics for TNBC are required, but this is still challenging.

Methionine addiction is a fundamental and general hallmark of cancer (3). Methionine addiction, discovered by our laboratory, involves elevated methionine flux in cancer cells (4-6) due to elevated transmethylation-reactions (5, 7). Methionine overuse by cancer cells is known as the Hoffman effect, analogous to the Warburg effect of glucose overuse by cancer cells (8). Methionine restriction results in free-methionine and S-adenosylmethionine (SAM) depletion (6, 7, 9), and selective cell-cycle arrest in the S/G_2 phase in cancer cells (10, 11). Methionine addiction and DNA hypomethylation in human cancer, also discovered in our laboratory (12), are related (13, 14). Because methionine addiction is tightly linked to other features of cancer (15) and is a general phenomenon of cancer, it can be an important target for cancer treatment. Recently, Jeon *et al*. (16) observed that methionine restriction with a low-methionine diet inhibited TNBC metastasis in mouse models.

Recombinant methioninase (rMETase), a purified methionine-cleaving enzyme, can target methionine addiction and can be used to treat any cancer by methionine restriction which selectively traps the cells in the S/G_2-phase of the cell cycle, where they are susceptible to most cytotoxic chemotherapy and can be successfully eradicated (17, 18). METase already has shown anticancer efficacy on many solid tumors, for example, sarcoma (19, 20), malignant melanoma (21, 22), pancreatic

Correspondence to: Robert M. Hoffman, PhD, AntiCancer Inc, 7917 Ostrow St, San Diego, CA, 92111, U.S.A. Tel: +1 8586542555, Fax: +1 8582684175, e-mail: all@anticancer.com; Hye In Lim, MD, Department of Surgery, Samsung Changwon Hospital, Sungkyunkwan University School of Medicine, 158, Paryong-ro, Masanhoewon-gu, Changwon-si, Gyeongsangnam-do, 51353, Republic of Korea. Tel: +1 8582096332, e-mail: vastprogress@naver.com

Key Words: Recombinant methioninase, PDOX, patient-derived orthotopic xenograft, TNBC, triple-negative breast cancer, resection, recurrence, inhibitor.

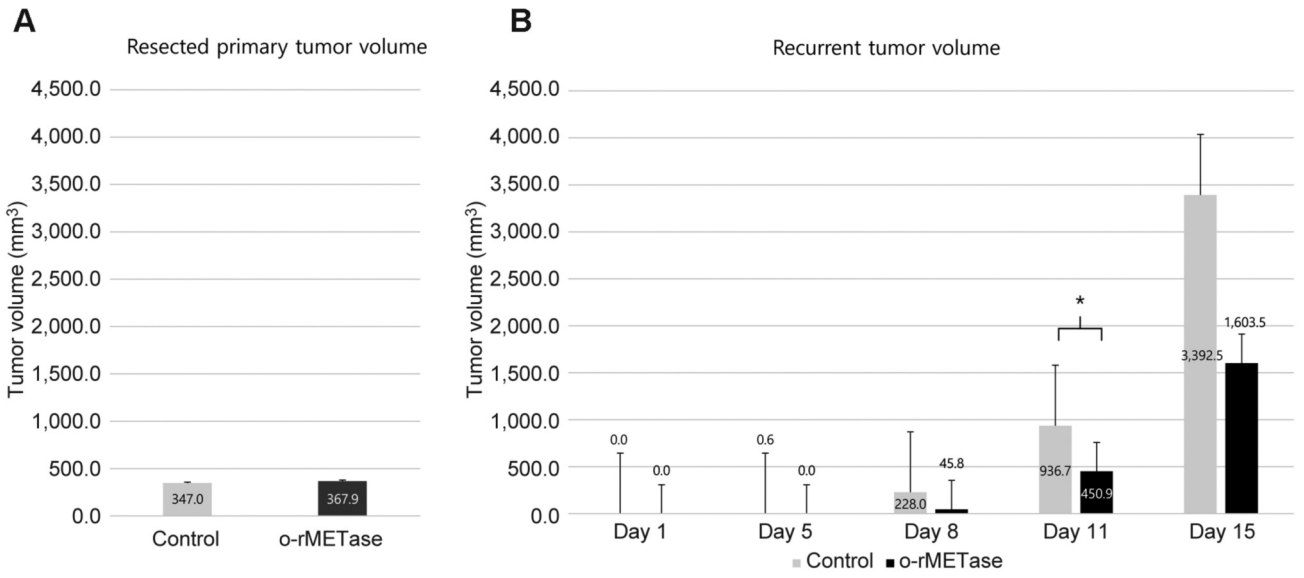

Figure 1. *A. Comparison of resected primary tumor volume measured after resection. B. Time course of recurrent tumor-volume changes. Error bars show the standard error of the mean (SEM). *p<0.05; o-rMETase: oral recombinant methioninase.*

cancer (23), and colon cancer (24, 25) *in vivo* and *in vitro*. But there has been a lack of studies on TNBC with METase.

We developed a patient-derived orthotopic xenograft (PDOX) (26-28) model of breast cancer in 1993 (29) to identify optimal therapy for individual patients and other laboratories have followed the model (30, 31).

In the present study, we evaluated the efficacy of orally-administered rMETase (o-rMETase) on a PDOX model of aggressive TNBC.

Materials and Methods

Mice. Athymic nu/nu nude female mice (AntiCancer Inc., San Diego, CA, USA), 4-6 weeks old, were used in this study. All animal studies were carried out with an AntiCancer Institutional Animal Care and Use Committee (IACUC)-protocol specifically approved for this study and according to the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Animals under Assurance Number A3873–1. Housing, diet, anesthesia of animals have been described in detail in a previous study (32).

o-rMETase production and formulation. An rMETase high-expression clone was engineered into E.coli. o-rMETase was purified in 3 steps including fermentation, purification and formulation. The fermentation procedure of the host *E.coli* cells, the purification protocol and formulation of rMETase have been previously described (33).

Patient-derived TNBC and establishment of PDOX. A 74 year-old female patient was diagnosed with TNBC in the right breast. She underwent breast-conserving surgery with sentinel lymph-node biopsy in the Department of Surgery, Samsung Medical Center (SMC), Seoul, Republic of Korea. The invasive ductal carcinoma

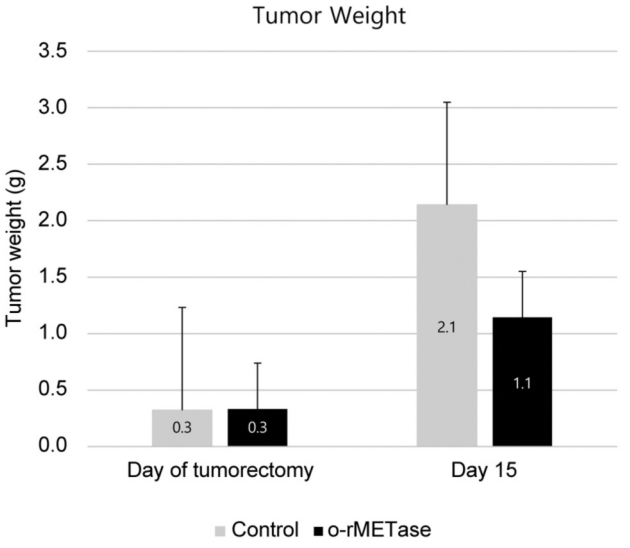

Figure 2. *Comparison of resected and recurrent tumor weight between the control and o-rMETase groups. Tumor weight was measured on the day of tumorectomy and on day 15 after tumorectomy. Error bars show standard error of the mean (SEM). o-rMETase: oral recombinant methioninase.*

tumor was 2.4 cm of histologic grade 3 and the Ki-67 value was 80.35%. There were neither regional nor distant metastasis.

Written informed consent was obtained from the patient, and the Institutional Review Board (IRB) of SMC approved this experiment. We previously established a PDOX model with the fresh resected tumor specimen from this patient, which was first implanted subcutaneously in nude mice. The method of establishing the PDOX

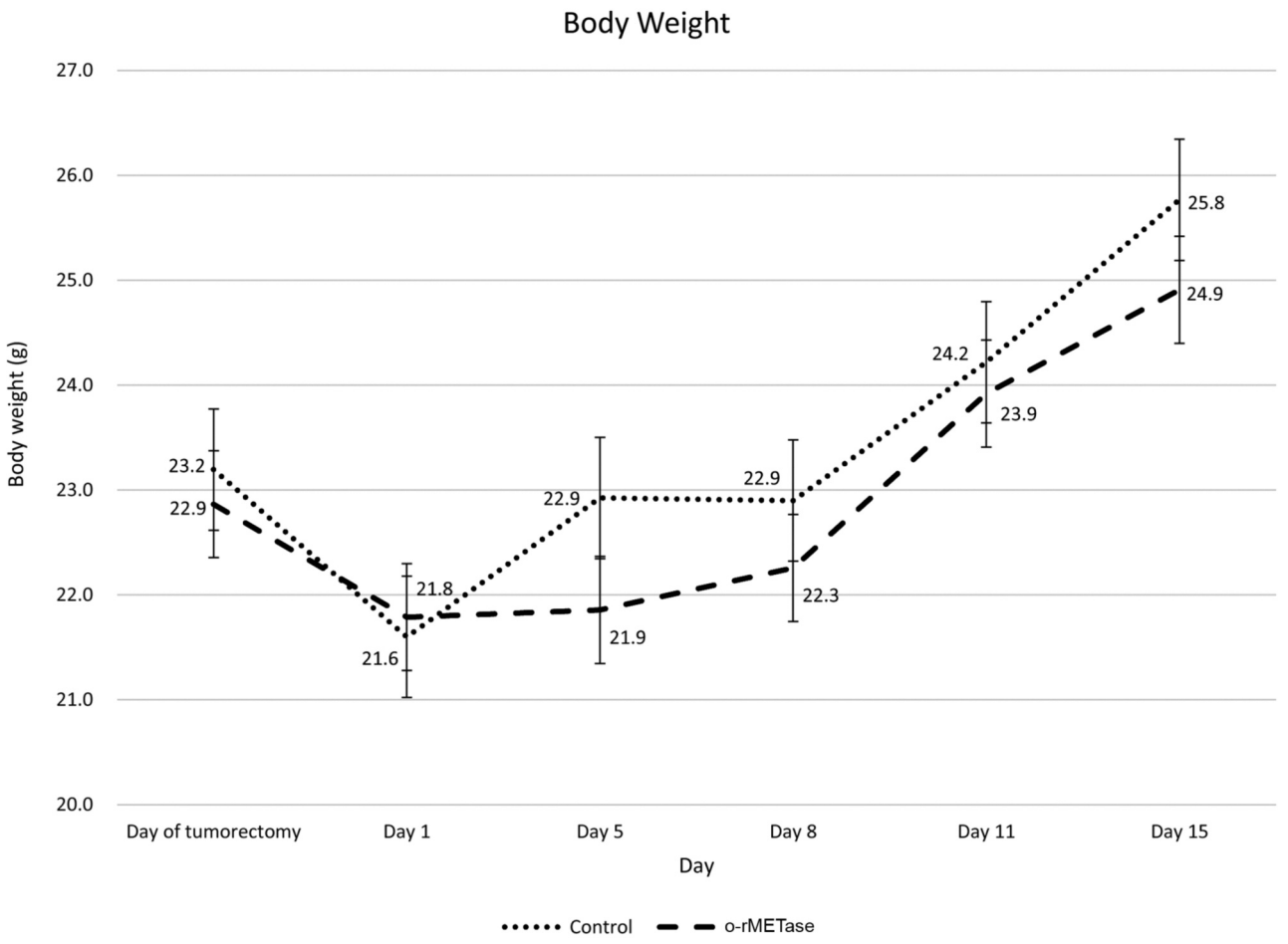

Figure 3. *Body weight of treated and untreated PDOX mice. Body weight was measured twice a week. Error bars show the standard error of the mean (SEM). PDOX: Patient-derived orthotopic xenograft; o-rMETase: oral recombinant methioninase.*

model using surgical orthotopic implantation (SOI) into the right 4th inguinal mammary fat pad has been previously reported (32).

Treatment dose and schedule. Two weeks after SOI, TNBC PDOX mouse models underwent tumorectomy with grossly-negative surgical margins. Two days after tumorectomy, the mice were randomized into two groups and treatment was started (day 1). G1, untreated control [n=10, PBS 0.1 ml, per os (*p.o.*), twice a day, 14 consecutive days] and G2, o-rMETase (n=9, 50 units, *p.o.*, twice a day, 14 consecutive days). All mice were humanely sacrificed on the following day of the last treatment.

Tumor length, width, and mouse body weight were measured twice a week. Tumor volume was calculated using the following formula: Tumor volume (mm^3)=length (mm)×width (mm)×width (mm)×1/2 (34).

Results

On the day of tumorectomy, the mean resected primary tumor volumes were 347.0 mm^3 in the control group and 367.9 mm^3 in the o-rMETase group (Figure 1A). Mean primary tumor weights were the same, 0.3 g, in both groups (Figure 2). Two days after tumorectomy, o-rMETase treatment was started (day 1). By day 11, all mice had recurrent tumors and mean recurrent tumor volumes were 936.7 mm^3 in the control group and 450.9 mm^3 in the o-rMETase group ($p<0.05$, Figure 1B). On day 15, mean recurrent tumor volumes were 3392.5 mm^3 in the control group and 1603.5 mm^3 in the o-rMETase group. Tumor weights were 2.1 g in the control group and 1.1 g in the o-rMETase group (Figure 2). There was no significant difference in body weight between the two groups (Figure 3).

Discussion

TNBC is a recalcitrant cancer with a very poor survival rate for patients with recurrent disease. A pattern of loco-regional recurrence in patients with TNBC is characterized by a rapidly rising recurrence rate in the first 2 years following diagnosis (35). TNBC has limited treatment options to inhibit recurrence, following conventional adjuvant chemotherapy.

In the present study, there was reduced recurrent tumor growth in the o-rMETase-treated group, compared to the control. These results suggest the use of a higher dose of methioninase and combination with chemotherapy to further control loco-regional recurrence. Our previous studies have indicated that the combination of chemotherapy with methionine restriction is often highly effective (18-25, 36).

The present study has an important implication, since this is the first *in vivo* study of o-rMETase using a breast cancer PDOX model. The results indicate that o-rMETase can be an effective treatment to inhibit recurrent tumor growth in TNBC, as a cancer-specific metabolism-targeted therapy. Our previous experiments have shown o-rMETase holds much promise (19-25, 36-38), as it is an oral drug, despite being a large tetrameric protein, with no known side effects. We recently published a study on the long-term treatment with o-rMETase of a patient with bone-metastatic prostate cancer (38). Treatment twice a day for a period over three months resulted in a 70% reduction in the patient's PSA with no observable side effects. It is hoped o-rMETase can be introduced to the clinic for patients with TNBC in the nearest time. Recently, studies have been published claiming the novelty of methionine addiction (39, 40), about which we published long ago (3-7, 9-11, 15, 41-44).

Efforts to identify novel effective therapeutics for TNBC are ongoing. But they are still challenging, and studies on methionine addiction in TNBC are highly promising.

Methionine addiction is considered cancer-specific re-programmed metabolism, which is as an important target for cancer diagnosis and therapy. Methionine deprivation had a strong inhibitory effect on the migration and invasion of TNBC cell lines (16), and produced a targetable vulnerability in TNBC by enhancing TNF-related apoptosis-inducing ligand receptor-2 (TRAIL-2) expression (45). These results suggested that targeting cancer-specific metabolism with o-rMETase has high clinical potential (38) compared to injectable rMETase (46). Targeting methionine and methylation can be a new effective modality for treatment of TNBC. Our future studies will involve administration o-rMETase in combination with or following first line TNBC chemotherapy as well as other treatment, both approved and experimental. Targeting a central aspect of metabolism such as that of methionine has much more potential for cancer therapy than targeting peripheral metabolism (47).

Conflicts of Interest

AntiCancer Inc. uses PDOX models for contract research. QH and YT are employees of AntiCancer Inc. HIL, KH, JY, RMH are or were unsalaried associates of AntiCancer Inc. There are no other competing financial interests.

Authors' Contributions

HIL performed experiments and wrote the paper. KH provided conceptual advice. JY provided technical advice. GH and YT provided o-rMETase. HJC and SJN provided the patient's tumor. MB reviewed the manuscript. RMH provided conceptual advice and edited the paper.

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