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# Response of Triple-negative Breast Cancer Liver Metastasis to Oral Recombinant Methioninase in a Patient-derived Orthotopic Xenograft (PDOX) Model

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**Abstract.** *Background/Aim: The aim of this study was to establish a patient-derived orthotopic xenograft (PDOX) mouse model of liver metastasis of triple-negative breast cancer (TNBC) and examine the efficacy of oral recombinant methioninase (o-rMETase) on the liver metastasis. Materials and Methods: TNBC from a patient was implanted in the left hepatic lobe of nude mice to simulate liver metastasis in a PDOX model. Ten days later, all mice underwent laparotomy to measure tumor size and were randomized to three groups: control; o-rMETase 100 U once daily (qd); and o-rMETase 200 U qd. After 9 days of treatment, all mice were sacrificed. Results: At the end of the treatment period for the liver metastasis, the size of liver metastases was 372.6 $mm^3$ in the control group; 160.0 $mm^3$ in the o-rMETase 100 U group; and 245.3 $mm^3$ in the o-rMETase 200 U group. All mice had ascites and 12 out of 14 mice in all groups had mesenteric lymph-node metastasis, as re-metastasis. The mean body-condition score was 1.5 in the control group; 2.4 in the o-rMETase 100 U group; and 2.6 in the o-rMETase 200 U group (control group vs. o-rMETase 200 U group, $p<0.05$). Conclusion: The TNBC liver metastasis was highly aggressive resulting in re-metastasis and ascites. o-rMETase tended to inhibit the liver metastasis and significantly improved the mouse body-condition score. This new PDOX model of TNBC liver metastasis will be useful for identifying effective agents for this recalcitrant disease.*

Triple-negative breast cancer (TNBC) accounts for about 15~20% of breast cancers. TNBC grows and spreads faster and has higher risk of early relapse with visceral metastasis compared to other subtypes of breast cancer. The 5-year relative survival rate of TNBC with distant metastasis is only 11% and there is rapid progression from distant recurrence to death (1). Because TNBC with distant metastasis has a higher frequency of progression, efforts to identify effective treatments for metastatic TNBC are necessary.

Cancer cells are methionine addicted due to enhanced overall rates of transmethylation and therefore depend on high levels of methionine compared to normal cells (2-5). The high methionine/methylation flux of cancer cells is known as the Hoffman effect (6), analogous to the Warburg effect of glucose overuse by cancer cells. Our laboratory discovered methionine-addiction of cancer (2-5). We have studied this phenomenon for almost 50 years and have concluded that methionine-addiction is the most fundamental and general hallmark of cancer (3-5). Methionine-addiction is tightly linked to global epigenetic changes in cancer controlled by methylation events (7) and is a promising target of cancer treatment.



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*Key Words:* PDOX, patient-derived orthotopic xenograft, TNBC, triple-negative breast cancer, liver metastasis, re-metastasis, lymph node, ascites, oral recombinant methioninase, treatment.

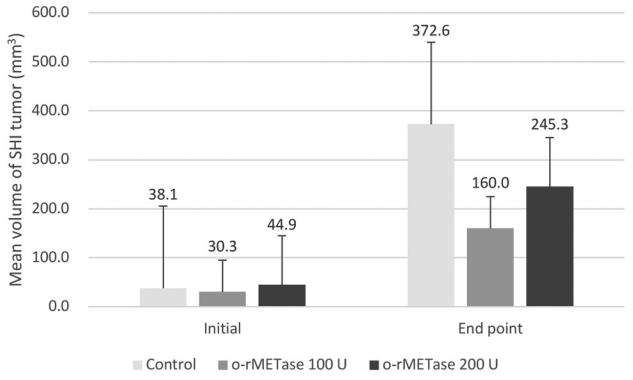


Figure 1. *Efficacy of o-rMETase on TNBC liver metastasis. SHI: surgical hepatic implantation, o-rMETase: oral recombinant methioninase, TNBC: triple negative breast cancer. Error bars show standard error of the mean (SEM).*

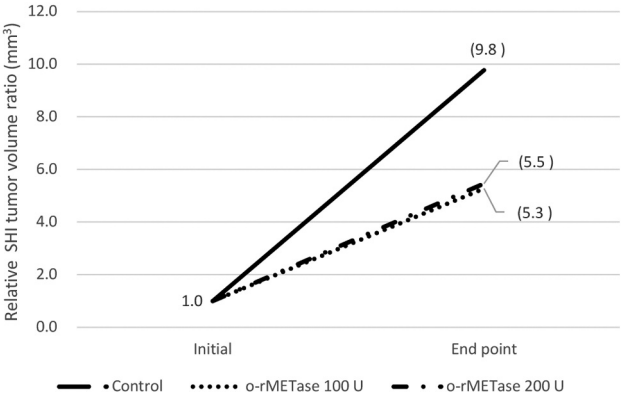


Figure 2. *Comparison of relative tumor volume of SHI tumor with and without o-rMETase treatment. SHI: surgical hepatic implantation, o-rMETase: oral recombinant methioninase. Relative tumor volume (values in parentheses) is the ratio of the tumor volume at the endpoint compared to the initial tumor volume.*

Methionine restriction selectively traps cancer cells in the $S/G_2$-phase of the cell cycle (8), where they are susceptible to most cytotoxic chemotherapy and can be successfully eradicated (2, 9). Recombinant methioninase, administered orally (o-rMETase), has shown efficacy in many solid tumors, for example, sarcoma (10-11), pancreatic cancer (12), colon cancer (13, 14), and malignant melanoma (15, 16) in patient-derived orthotopic xenograft (PDOX) mouse models, by restricting methionine.

We previously established liver-metastasis models of patient-derived colon cancer in nude mice (17-19). Lymph-node metastasis was found at the site of drainage of the liver: celiac, portal and mediastinal lymph nodes which originated from the liver metastasis, and not, as previously thought, from primary colon cancer. We suggested the concept of re-metastasis which means "metastasis of metastases" (18).

We previously established a patient-derived orthotopic xenograft (PDOX) model of highly-aggressive TNBC transplanted to the mammary fat pad of nude mice (20). In the present study, we established an aggressive TNBC liver-metastasis model by surgical hepatic implantation (SHI) in nude mice and evaluated the efficacy of o-rMETase.

## Materials and Methods

*Mice*. Athymic nu/nu nude, 4-6 weeks old female mice (AntiCancer Inc., San Diego, CA, USA), were used in this study. All animal studies were carried out with an AntiCancer Institutional Animal Care and Use Committee (IACUC)-protocol approved for this study and according to the procedures and principles 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 (20).

*o-rMETase production and formulation*. The L-methionine-γ-deamino-α-mercaptomethane-lyase gene from *Pseudomonas putida* has been cloned in *E. coli*. o-rMETase is produced in three steps from the recombinant *E. coli*, 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 (21).

*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 at the Department of Surgery, Samsung Medical Center (SMC), Seoul, Korea. The tumor (2.4 cm) was an invasive ductal carcinoma with histological grade 3 and an 80% Ki-67 value. Regional and distant metastasis were not detected.

Written informed consent was obtained from the patient, and the Institutional Review Board (IRB) of SMC approved this experiment. We established the TNBC in nude mice as previously described (20). In the present study, when subcutaneously-grown tumors reached 10 mm in diameter, they were harvested and cut into approximately 1 $mm^3$ size fragments. For surgical hepatic implantation (SHI), the abdomen was sterilized with 70% alcohol and a left para-median incision was made under anesthesia. After the left lobe of the liver was exteriorized, a shallow 2 mm-length incision was made with an Iris surgical scissors on the serosa of the left-lateral lobe. The tumor fragment was inserted into this incision and followed by bleeding control with compression. The abdominal wall was closed with a 6-0 nylon suture.

*Treatment dose and schedule*. Ten days after SHI, mice underwent laparotomy to observe the tumor size on the liver. The mice were randomized into three groups of equivalent average tumor size: G1, untreated control [n=4, PBS 0.1 ml, per os (p.o.), twice a day, 9 consecutive days]; G2, 100 U o-rMETase treatment (n=5, 50 units, p.o., twice a day, 9 consecutive days); and G3, 200 U o-rMETase treatment (n=5, 100 units, p.o., twice a day, 9 consecutive days). Treatment was started two days after laparotomy. All mice were humanely sacrificed on the following day of the last treatment.

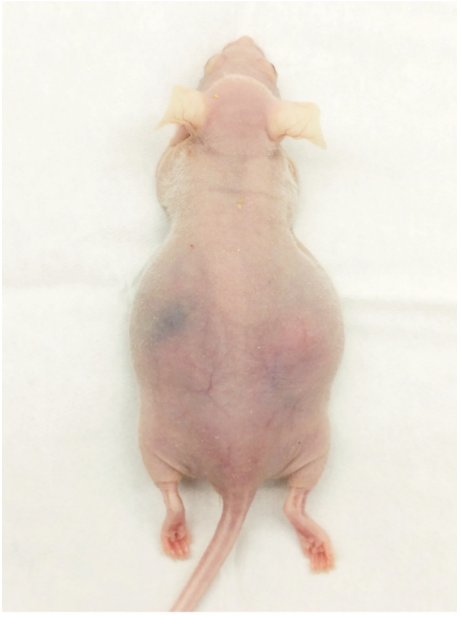

Figure 3. *Mouse with ascites in control group.*

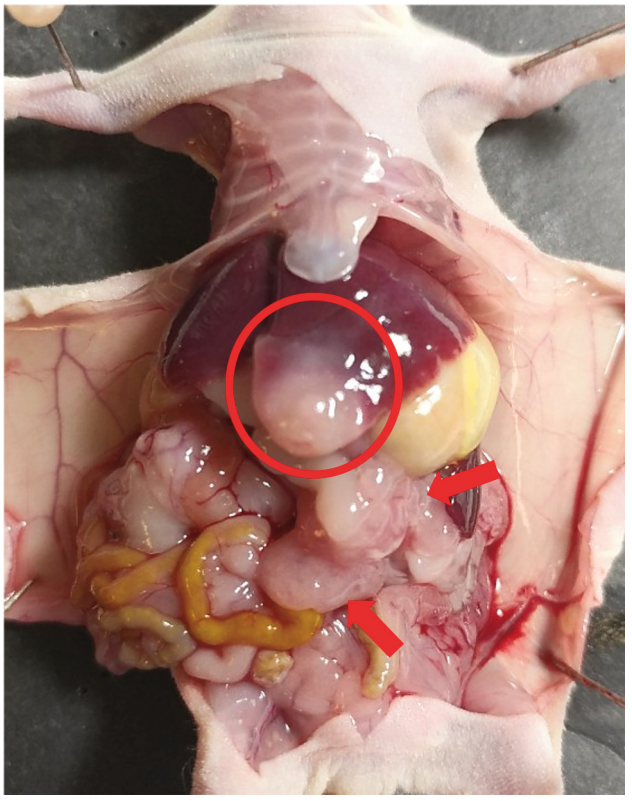

Figure 4. *Tumor and lymph-node re-metastasis in TNBC SHI model on the day following the last treatment. Red circle is SHI tumor and red arrows are lymph-node re-metastasis. TNBC: Triple-negative breast cancer, SHI: surgical hepatic implantation.*

Mouse body weight was measured every day or two and tumor volume was measured on the day of laparotomy and on the day of sacrifice. Tumor volume was calculated using the following formula: Tumor volume ($mm^3$)=length (mm) × width (mm) × width (mm) ×1/2 (22). Mouse condition was determined using body-condition scoring (BCS) (23), and behavior and appearance scoring (24).

## Results

We implanted tumors to the liver of 22 mice using SHI. On the day of laparotomy before treatment, 14 mice had liver tumors and the mean tumor volume was 38.1 $mm^3$ in the untreated control group; 30.3 $mm^3$ in the o-rMETase 100 U treatment group; and 44.9 $mm^3$ in the o-rMETase 200 U treatment group. After 9 days of treatment, the mean tumor volume of the liver was 372.6 $mm^3$ in the control group; 160.0 $mm^3$ in the o-rMETase 100 U treatment group; and 245.3 $mm^3$ in the o-rMETase 200 U treatment group (Figure 1). The tumor volume ratio at the end of the treatment period relative to beginning was 9.8 in the control group; 5.3 in the o-rMETase 100 U group; and 5.5 in the o-rMETase 200 U group (Figure 2).

All mice had ascites (Figure 3) and 12 out of 14 mice had mesenteric metastatic lymph-node metastasis (Figure 4), except one each in the control and o-rMETase 100 U group.

The mean behavioral and appearance scores were 5.5 in the control group; 9.6 in the o-rMETase 100 U group; and 10 in the o-rMETase 200 U group (Figure 5A). The mean BCS was 1.5 in the control group; 2.4 in the o-rMETase 100 U group; and 2.6 in the o-rMETase 200 U group at the end point (control group vs. o-rMETase 200 U treatment group, $p<0.05$) (Figure 5B).

There was no significant difference in body weight between groups, but o-rMETase 200 U may have prevented cachexia. (Figure 6).

Hematoxylin and eosin staining showed cancer cells in both the lymph-node and liver metastasis (Figure 7).

## Discussion

Mouse models of breast-cancer metastasis to the liver use human breast-cancer cell lines. Since liver metastasis rarely occurs in subcutaneous or orthotopic xenograft models of breast cancer, intracardiac or intrasplenic injection is used, but these models are not specific and can cause concurrent metastases in other organs (25). We previously established liver-metastasis models of patient-derived colon cancer in nude mice using SHI (17-19) and discovered the phenomenon of re-metastasis (18). In the present study, we established a liver-metastasis model of patient-derived TNBC by SHI,

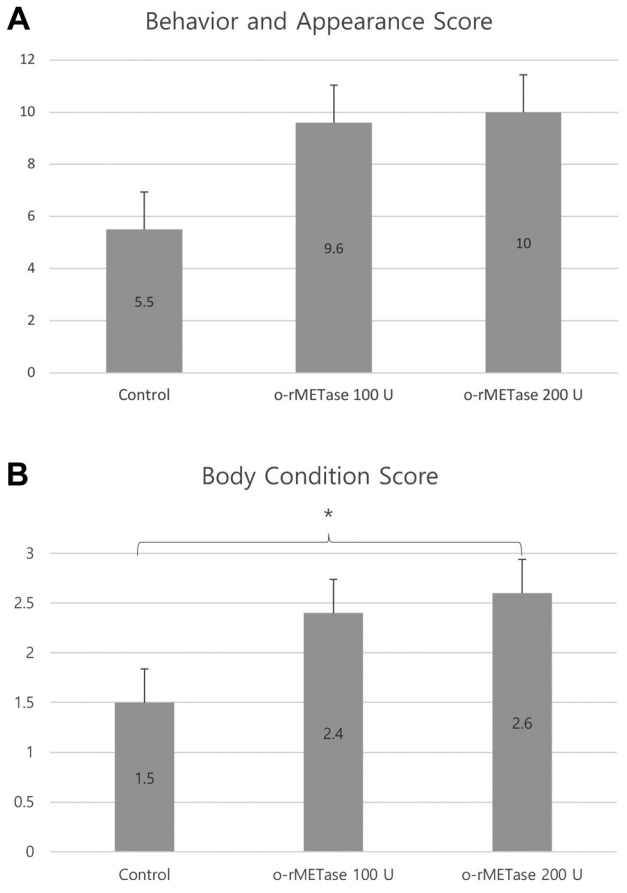


Figure 5. *Comparison of body scoring of control and o-rMETase-treated mice. (A) Behavioral and appearance score at the end point. This score is quantified appearance, natural behavior, provoked behavior and ranged from 1 to 13 (23). (B) BCS at the end point. BCS is quantified by the amount of flesh covering body protuberances and raged from 1 to 5 (24). Error bars show standard error of the mean (SEM). o-rMETase: Oral recombinant methioninase, BCS: body condition score, *p<0.05.*

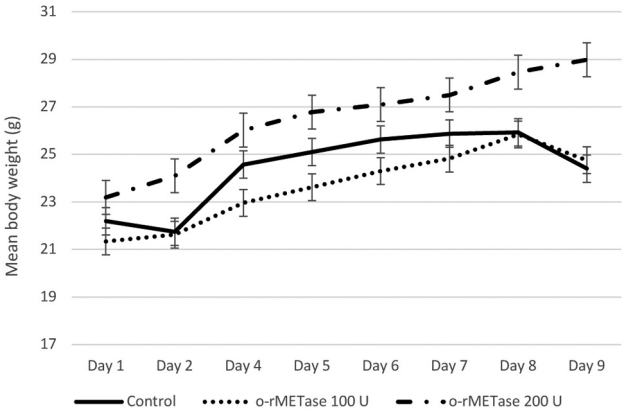


Figure 6. *Comparison of body weight over time during treatment. Error bars show the standard error of the mean (SEM).*

simulating liver metastasis. In this SHI model, we found mesenteric lymph-node metastasis following liver metastasis growth, as a result of re-metastasis. Re-metastasis should be considered clinically when we treat metastatic disease.

TNBC has an increased likelihood of distant recurrence compared to other types of breast cancer and the risk of distant recurrence peaks between 1 to 3 years after surgery. The time from recurrence to death is 9 months, significantly shorter than other types of breast cancer (1). Because TNBC has no targetable marker, such as hormonal receptors or human epidermal growth factor receptor-2 (HER-2), the treatment options for metastatic disease are limited and novel treatment is necessary.

Compared to normal cells, cancer cells require high levels of methionine to proliferate due to methioninase addiction (2-5). Methionine addiction is cancer-specific metabolism and distinguishes cancer cells from normal cells. Targeting cancer-specific altered methionine metabolism has potential as a clinical cancer treatment (9) and can be a new effective treatment modality of TNBC. Methionine-restriction has been studied in a TNBC mouse model by Jeon *et al.* (26) who reported that a low-methionine diet inhibited TNBC metastasis in mice. Strekalova *et al.* (27) reported that dietary methionine deprivation enhanced the anti-tumor efficacy of a humanized agonistic TNF-related apoptosis-inducing ligand receptor-2 (TRAIL-R2) monoclonal antibody by increasing TRAIL-R2 expression in a model of TNBC.

In the present study, o-rMETase trended to inhibit the growth of the SHI tumors and resulted in better mouse condition. These results suggest that o-rMETase has potential as a new effective modality for metastatic TNBC, especially since it can be administered orally without toxicity (28, 29).

Our future studies will involve administration o-rMETase in combination with conventional and experimental chemotherapy for TNBC metastases using PDOX mouse models (10-16, 29). o-rMETase has already shown promise in the clinic (28) and will soon be tested on TNBC patients, especially with liver metastasis. o-rMETase has previously shown efficacy to inhibit post-surgical recurrence of TNBC in a PDOX nude-mouse model (30). This new PDOX model of TNBC liver metastasis has potential to identify curative therapeutics for this recalcitrant disease, especially using o-rMETase instead of injectable rMETase which can cause immunological reactions (31), unlike o-rMETase (28), and will therefore, be suitable clinically in the near future for TNBC.

## Conflicts of Interest

None to be declared.

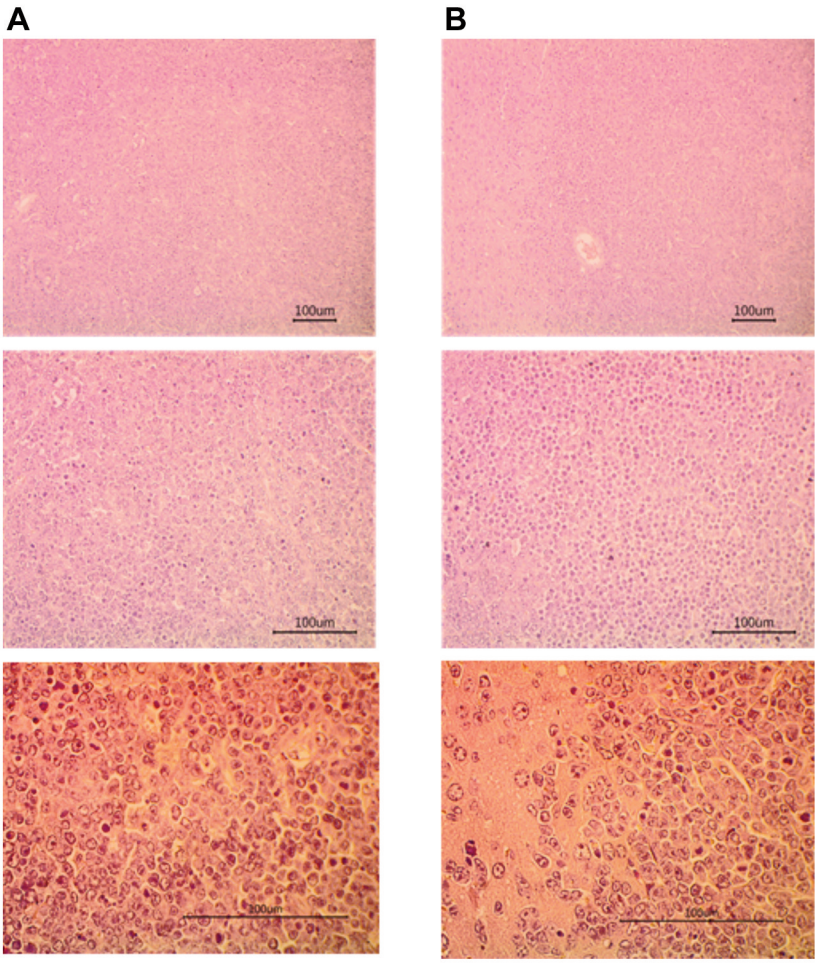


Figure 7. *Histology of PDOX TNBC. (A) H&E staining of mesenteric lymph-node re-metastasis. (B) H&E staining of SHI tumor. Upper row: ×100, middle row: ×200, lower row: ×400. PDOX: Patient-derived orthotopic xenograft; TNBC: triple-negative breast cancer; H&E: hematoxylin and eosin.*

## Authors' Contributions

HIL and RMH conceived the project. HJC, SJN and MB provided scientific advice for the project. HIL, JY, YS, HN, YT, and NS contributed to mouse-model establishment and obtained experimental data. QH and YT provided methioninase. HIL and RMH wrote and revised the manuscript.

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