Recombinant-methioninase-producing *Escherichia coli* Instilled in the Microbiome Inhibits Triple-negative Breast Cancer in an Orthotopic Cell-line Mouse Model

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Abstract. Background/Aim: Methionine restriction by diet and recombinant methioninase (rMETase) are effective for cancer therapy by themselves or combined with chemotherapy drugs. We previously showed that oral administration of rMETase-producing *Escherichia coli* JM109 (E. coli JM109-rMETase) can be installed in the mouse microbiome and inhibit colon-cancer growth in a syngeneic mouse model. In the present report, we investigated the efficacy of oral administration of E. coli JM109-rMETase in an orthotopic triple-negative breast cancer (TNBC) cell-line mouse model. Materials and Methods: First, we established orthotopic 4T1 mouse triple-negative breast cancer on an abdominal mammary gland in female athymic nu/nu nude mice aged 4-6 weeks. After tumor growth, 15 mice were divided into three groups of 5. Group 1 was administered phosphate-buffered saline (PBS) orally by gavage twice daily as a control; Group 2 was administered non-recombinant E. coli JM109 competent cells orally by gavage twice daily as a control; Group 3 was administered E. coli JM109-rMETase cells by gavage twice daily for two weeks. Tumor size was measured with calipers twice per week. On day 15, blood methionine level was examined using an HPLC method. Results: Oral administration of E. coli JM109-rMETase inhibited 4T1 TNBC growth significantly compared to the PBS and E. coli JM109 control groups. On day 15, the blood methionine level was significantly lower in the mice administered E. coli JM109-rMETase than in the PBS control. Conclusion: E. coli JM109-rMETase lowered blood methionine levels and inhibited TNBC growth in an orthotopic cell-line mouse model, suggesting future clinical potential against a highly recalcitrant cancer.

Methionine addiction is a fundamental and general hallmark of cancer, termed the Hoffman effect (1-4). Methionine addiction is due to excess transmethylation reactions in cancer cells. Unlike normal cells, cancer cells cannot survive without exogenous methionine (5-11). We have reported the efficacy of recombinant methioninase (rMETase) for various cancer types in vitro, in vivo, and in the clinic (12). Oral administration of rMETase (o-rMETase), is highly effective in mouse models of cancer and is promising in the clinic (13-19). We previously reported the efficacy of o-rMETase for triple-negative breast cancer (TNBC) using a patient-derived orthotopic xenograft model (20-22).

Recently, we showed the efficacy of rMETase-producing *E. coli* JM109 (E. coli JM109-rMETase) administered orally against the mouse colon-cancer cell line MC38 in a syngeneic mouse model (23). The present report shows that oral administration of E. coli JM109-rMETase can be instilled in the mouse gut and inhibit breast-cancer growth. Breast cancer is the most common type of cancer and the second-leading cause of death among women in the United States (24). Breast cancer has three main subtypes: hormone receptor (HR)-positive, human epidermal growth factor receptor 2 (HER2)-positive, and TNBC, according to molecular
markers, such as estrogen receptor (ER), progesterone receptor (PR), and HER2. TNBC has a worse prognosis compared to HR-positive or HER2-positive breast cancer (25, 26). The standard treatment for metastatic or recurrent TNBC is chemotherapy with single-agent taxanes or anthracyclines. Recently, the efficacy of the combination of chemotherapy with an immune checkpoint inhibitor, such as atezolizumab or pembrolizumab, has shown a median overall survival of less than 23 months (27, 28).

In the present report, we examined the efficacy of E. coli JM109-rMETase on the mouse TNBC cell line 4T1 using an orthotopic cell-line mouse model.

Materials and Methods

E. coli JM109-rMETase culture. The host strain for the expression of rMETase was E. coli JM109. The plasmid pATG3131, which contains the tetracycline (TC)-resistance gene, was used to clone the rMETase gene from P. putida into E. coli JM109 (29, 30). The resultant E. coli JM109-rMETase was pre-cultured in 5 ml Luria-Bertani (LB) liquid medium for 8 h at 37°C with TC (32 μg/ml). The preculture broth was placed in 400 ml culture medium with TC (32 μg/ml) and grown overnight. To induce the expression of rMETase, isopropyl-D-thiogalactopyranoside (IPTG) was administered at a final concentration of 0.3 mM for 4 h at 37°C. To estimate the concentration of live E. coli JM109-rMETase in the medium, the optical density (OD) at 600 nm was used. An OD600 of 0.7 corresponds to approximately 1.0x10⁹ colony-forming units (CFU)/ml of E. coli JM109-rMETase, correlating to manually counting colonies. Harvested E. coli JM109-rMETase was diluted with phosphate-buffered saline (PBS), 20% glycerin, and stored at 80°C before being administered to mice. Similar to E. coli JM109-rMETase, E. coli JM109 competent cells, not treated with TC or IPTG, were prepared (23).

Mice. The present study used female athymic nu/nu nude mice (AntiCancer Inc., San Diego, CA, USA) that were 4-6 weeks old. The mice were kept in a barrier facility with a HEPA-filtered rack and 12-h light/dark cycle. During this experiment, mice were fed autoclaved laboratory rodent food. The AntiCancer Institutional Animal Care and Use Committee approved the study. Every experiment followed the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. 2.0 criteria (31).

Cell culture. High-glucose Dulbecco’s-modified Eagle’s medium (DMEM), containing 10% fetal bovine serum (FBS) and 100 IU/ml penicillin/streptomycin was used to cultivate the 4T1 mouse triple-negative breast cancer cell line at 37°C in a humid environment containing 5% carbon dioxide.

Establishment of orthotopic tumors. Twenty female athymic nu/nu nude mice, aged 4-6 weeks were given an orthotopic injection of 10⁵ 4T1 cells in an abdominal mammary gland. One week after injection, orthotopic tumors were established.

Treatment study design. Mice implanted orthotopically with 4T1 were randomized into three groups of five mice each: Group 1: Untreated control given 100 μl phosphate buffered saline (PBS) orally twice daily (9 a.m. and 5 p.m.) for 14 days; Group 2: E. coli JM109 competent cells (10¹⁰/100 μl) were administered orally by gavage twice daily (9 a.m. and 5 p.m.) for 14 days; Group 3: E. coli JM109-rMETase cells (10¹⁰/100 μl) were administered orally by gavage twice daily (9 a.m. and 5 p.m.) for 14 days. To prevent plasmid shedding in Group 3, TC (0.5 g/l) was added to the drinking water of the mice. TC was added to Group 1 as a control. IPTG was only administered to Group 3 in order to induce the production of methioninase by E. coli JM109-rMETase in the gastrointestinal tract of the mice. Tumor volume and body weight were measured twice a week during the treatment. Tumor volume (mm³) was calculated with the following formula: Tumor volume (mm³)=length (mm) × width (mm) × width (mm) × 1/2.

Measurement of plasma-methionine concentration. Mouse blood samples were collected through tail bleeding between 9 and 10 a.m. on day 15 for Group 1 and Group 3. The quantification of methionine in the plasma was conducted using pre-column derivatization, followed by high-performance liquid chromatography (HPLC) separation, following a previously-established protocol (32).

Statistics. Statistical analyses were carried out using GraphPad Prism 9.4.0 software (GraphPad Software, Inc., San Diego, CA, USA). The unpaired t-test or Tukey-Kramer post hoc test were used for the parametric comparison between groups. The data are presented as the mean plus-minus the standard deviation. Significance was attributed to p-values that were less than or equal to 0.05.

Results

Antitumor efficacy of oral E. coli JM109-rMETase. The growth of 4T1 tumors in athymic nu/nu nude mice was significantly suppressed by E. coli JM109-rMETase when compared to the PBS-treated control or E. coli JM109 competent cells that do not produce rMETase (p=0.0034 for the PBS control vs. E. coli JM109-rMETase; p=0.0113 for E. coli JM109 competent-cells control vs. E. coli JM109-rMETase) (Figure 1).

Body-weight change. Figure 2 shows the body-weight change of each group. No significant change in any group confirms the tolerability of 10¹⁰/100 μl E. coli JM109 for nude mice.

Methionine level. On day 15, the mean methionine level of Group 1 and Group 3 was as follows: E. coli JM109 competent-cells control group: 85.3 μM; E. coli JM109-rMETase group: 60.5 μM (p=0.0133), which is approximately a 30% reduction (Figure 3).

Discussion

We previously showed the efficacy of orally-administered E. coli JM109-rMETase against the mouse colon-cancer cell line MC38 in a syngeneic mouse model (23). The present study showed that E. coli JM109-rMETase is also effective against mouse TNBC. This bacterial therapy reduced blood methionine levels by approximately 30%, which is consistent with the efficacy of oral rMETase (33).
*E. coli* JM109-rMETase acts in the gut, and is also instilled in the microbiome and produces rMETase due to IPTG in the drinking water (23). The rMETase produced by the bacteria degrades methionine. Also, deoxycholic acid from the liver may lyse the bacteria, thereby releasing methioninase into the gut. Since intestinal epithelial cells consume significant amounts of methionine, approximately 20-30% of methionine is metabolized during absorption (34). Therefore, it is reasonable to target the intestine for methionine restriction with *E. coli* JM109-rMETase.

In a recent study, the genetically modified strain of *E. coli* Nissle was employed to metabolize methionine through the methionine decarboxylase pathway (35). The findings revealed a decrease of 32% in blood methionine levels in mice, and 25-26% in humans. This concept is a methionine-consuming recombinant pro-biotic engineered with methionine decarboxylase. This pro-biotic was used to treat homocystinuria by lowering the amount of methionine in the blood of humans.

In conclusion, bacterial therapy using *E. coli* JM109-rMETase showed growth-inhibitory efficacy against a mouse...
TNBC 4T1, in nude mice. As demonstrated by E. coli JM109-rMETase in the present and previous reports (23), recent advances in manipulating the gut microbiome to reduce methionine indicate the future potential for treating recalcitrant cancer. Methionine-restriction therapy is effective since it targets the fundamental basis of cancer, methionine addiction (11-1, 36-42).

The US National Cancer Institute has been allocated $160 billion since 1971, yet the mean survival of TNBC patients is less than 23 months (27, 28). Methionine-restriction therapy should increase TNBC survival.

Conflicts of Interest

The Authors declare no competing interests regarding this work.

Authors' Contributions

YK and RMH wrote the article. QH, SM, KM, MB and TT reviewed the article.

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