in vivo *35*: 3067-3071 (2021)
doi:10.21873/invivo.12602

Salmonella typhimurium A1-R Exquisitely Targets and Arrests a Matrix-producing Triple-negative Breast Carcinoma in a PDOX Model

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Abstract. *Background/Aim: Triple-negative matrix-producing breast carcinoma (MPBC) is rare, recalcitrant, and highly aggressive. The present study aimed to determine the efficacy of tumor-targeting leucine-arginine auxotroph Salmonella typhimurium (S. typhimurium) A1-R on a triple-negative MPBC in a patient-derived orthotopic xenograft (PDOX) model. Materials and Methods: The PDOX MPBC model was established in the left second mammary gland of nude mice by surgical orthotopic implantation (SOI). PDOX models were randomized into two groups when the tumor volume reached over 70 mm^3: a control group (n=6); and a tumor-targeting S. typhimurium A1-R group (n=7), [intravenous (i.v.) injection of S. typhimurium A1-R via the tail vein, weekly, for two weeks]. All mice were sacrificed on day 14. Tumor volume and body weight were measured once per week. Results: S. typhimurium A1-R exquisitely targeted and arrested the growth of the MPBC PDOX compared to the control group (p=0.017). Conclusion: S. typhimurium A1-R has future clinical potential for triple-negative MPBC patients.*

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Key Words: PDOX, patient-derived orthotopic xenograft, triple-negative breast cancer, matrix-producing breast carcinoma, *Salmonella typhimurium* A1-R, tumor targeting, efficacy.

Triple-negative matrix-producing breast carcinoma (MPBC) is defined by the absence of estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2) and the production of a cartilaginous or osseous matrix (1, 2). Effective therapy for triple-negative MPBC has not been established due to its rarity (3).

Our laboratory pioneered the patient-derived orthotopic xenograft (PDOX) model 30 years ago by establishing the technique of surgical orthotopic implantation (SOI) (4, 5). We have shown that the PDOX model retains the histopathological/molecular and metastatic characteristics of the original tumor after SOI unlike the subcutaneous PDX mouse model (5-7). We previously showed eribulin regressed triple-negative MPBC in PDOX mouse models (8, 9).

Leucine-arginine auxotroph *Salmonella typhimurium* (*S. typhimurium*) A1-R, expressing green fluorescent protein (GFP), accurately targets tumors and does not grow well in normal tissue due to its leucine–arginine auxotrophy (10-12). *S. typhimurium* A1-R inhibited prostate (10, 12), breast (11, 13, 14), lung (15, 16), pancreatic (17-19), ovarian (20, 21), stomach (22), and cervical cancer (23), as well as sarcoma (24-26), melanoma (27), and glioma (28).

In the present report we demonstrate that *S. typhimurium* A1-R exquisitely targets and arrests a triple-negative MPBC in a PDOX mouse model.

Materials and Methods

Mouse studies. Ethical approval was obtained from the ethics committee of the AntiCancer Institutional Animal Care and Use Committee under the National Institutes of Health Guide Assurance Number A3873-1(8). All experiments were conducted in compliance with Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines 2.0.

Patient tumor. The patient tumor was provided as a discarded pathology specimen by Kawasaki Medical School, Japan, following Research Ethics Committee of Kawasaki Medical School and Hospital approval. All experiments were carried out in accordance with the Declaration of Helsinki and regulations for human studies, and informed consent was obtained from the patient. The patient MPBC tumor was previously established to grow subcutaneously in nude mice. Subcutaneously-grown tumor was harvested and minced into 3 mm^3 fragments and orthotopically implanted into the 2nd left mammary gland of nude mice as previously described (8).

Preparation of Salmonella typhimurium A1-R. S. typhimurium A1-R-GFP (AntiCancer, Inc., San Diego, CA, USA) was produced as described in our previous publications (10-28): Bacteria were incubated in Luria-Bertani (LB) medium containing ampicillin at 37˚C overnight with shaking. The bacteria were then diluted 10-fold in LB medium containing ampicillin and incubated for 3 hours under the same conditions. Bacteria were washed with phosphate-buffered saline (PBS), suspended in PBS, and injected into nude mice *via* the tail vein.

Treatment protocol for the triple-negative MPBC PDOX model. The detailed experimental schema is shown in Figure 1. The triple-negative MPBC PDOX models were randomized into two groups when the tumor volume was over 70 mm^3: Control: untreated control mice; A1-R: *S. typhimurium* A1-R-GFP treated mice [*i.v.*, 5×10^7 colony-forming units (CFU) *S. typhimurium* A1-R -GFP in 100 μl PBS (weekly, two weeks)]. Each group comprised six and seven mice, respectively. Tumor size and body weight were measured once a week. Tumor volume was calculated using the following formula: tumor volume (mm)=length (mm) × width (mm) × width (mm) × 1/2. All mice were sacrificed on day 14.

Fluorescence imaging of S. typhimurium A1-R-GFP in the triple-negative MPBC PDOX. Fluorescence images were obtained and analyzed using the UVP ChemStudio (Analytik Jena, Thuringia, Germany).

Statistical analysis. GraphPad Prism 8.4.3 (GraphPad Software, Inc., San Diego, CA, USA) was used for statistical calculations. The Student's *t*-test was used to compare groups. Data are the mean values±SD. $p\leq0.05$ is defined as statistically significant.

Results

Exquisite targeting of the triple-negative MPBC PDOX by S. typhimurium A1-R-GFP. Twenty-four hours after infection of triple-negative MPBC PDOX with *S. typhimurium* A1-R -GFP, the bacteria selectively targeted the tumor, as demonstrated by GFP fluorescence in the tumor but not in the liver or spleen of the triple-negative MPBC PDOX mouse model (Figure 2).

Efficacy of S. typhimurium A1-R - GFP on the triple-negative MPBC PDOX. S. typhimurium A1-R arrested the growth of the triple-negative MPBC ($p=0.017$). The final tumor volume was obtained on treatment-day 14. Average tumor volume of the control group was 205±70 mm^3 and in the *S. typhimurium* A1-R -GFP group 112±37 mm^3 (Figure 3).

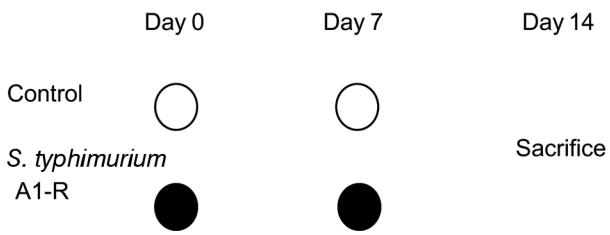

Figure 1. *Experimental schema. S. typhimurium A1-R-GFP was injected into the tail vein of triple-negative matrix-producing breast carcinoma PDOX nude mice [5×10^7 colony-forming units (CFU) per 100 μl of PBS].*

No effect of S. typhimurium A1-R-GFP on body weight of the triple-negative MPBC PDOX mouse model. To determine whether S. typhimurium *A1-GFP* had a gross adverse effect, the mouse body weight was measured at pre-treatment and post-treatment. The final body weight on treatment-day 14 was 24.7±0.9 g for the control group and 24.1±3.1 g for the *S. typhimurium* A1-R - GFP group. There were no significant differences in the body weight between the two groups on day14 ($p=0.73$) (Figure 4), which suggests that *S. typhimurium* A1- R-GFP had no obvious side effects.

Discussion

In the present study, *S. typhimurium* A1-R-GFP accurately targeted the triple-negative MPBC in a PDOX model and arrested its growth.

Kusafuka *et al.* reported the frequency of MPBCs is only 0.2% of all breast cancers (2). MPBC is usually triple-negative breast cancer (TNBC) and has a high proliferative activity, indicated by high histological grade, high Ki-67 index, and high level of p53 expression (1, 29). Shimada *et al.* reported that the mean Ki-67 index of non-TNBC MPBCs (45%) was higher than that of TNBCs (36%) (3).

Due to its rarity, there are only a few clinical trials for MPBC and they showed poor efficacy (3, 30, 31). Therefore, novel effective therapy is urgently needed for MPBC patients. Our previous studies of triple-negative MPBC and common triple-negative breast cancer (TNBC) PDOX mouse models showed eribulin was effective (8, 9).

Bacterial therapy has gained popularity as a cancer immunotherapy in recent years (32). *Salmonella, Clostridium,* and other bacterial genera have been shown to control tumor growth and promote survival in animal models (32). *S. typhimurium* A1-R is an auxotrophic leucine-arginine facultative-anaerobic *S. typhimurium* strain, which selectively targets and proliferates in tumors of all types due to, at least in part, its nutritional needs, which appear to be satisfied in

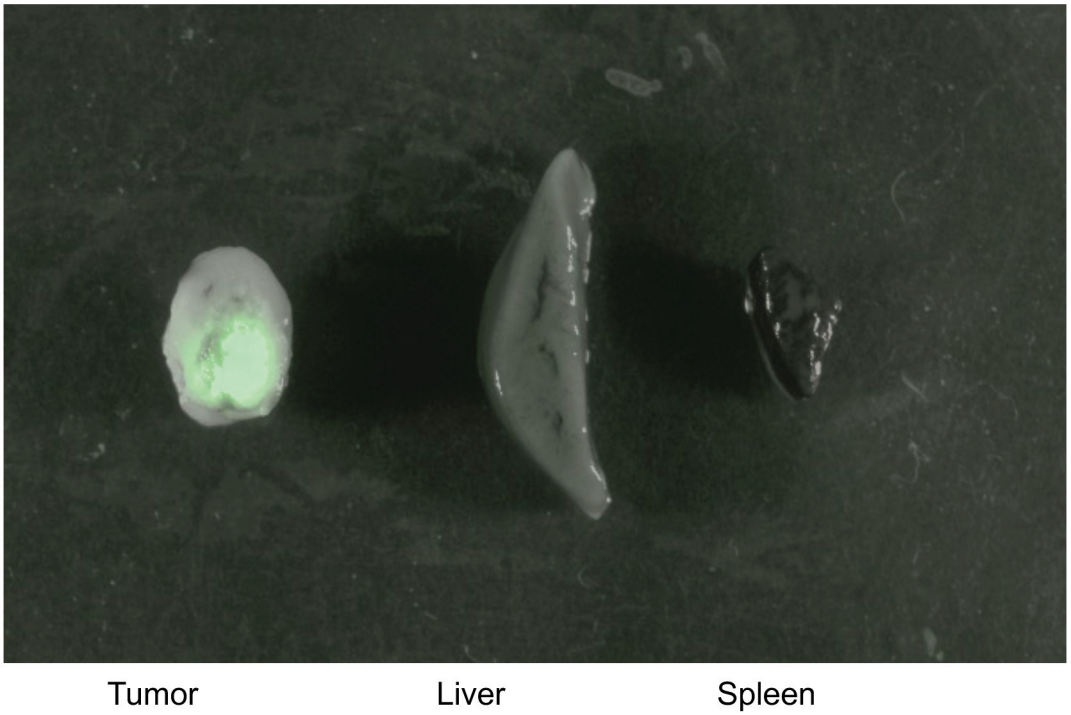

Figure 2. *Tumor targeting by S. typhimurium A1-R-GFP. Selective tumor targeting of S. typhimurium A1-R-GFP to the triple-negative matrix-producing breast carcinoma (MPBC) PDOX. Imaging was performed with the UVP ChemStudio (Analytik, Jena, Germany). Representative images are shown.*

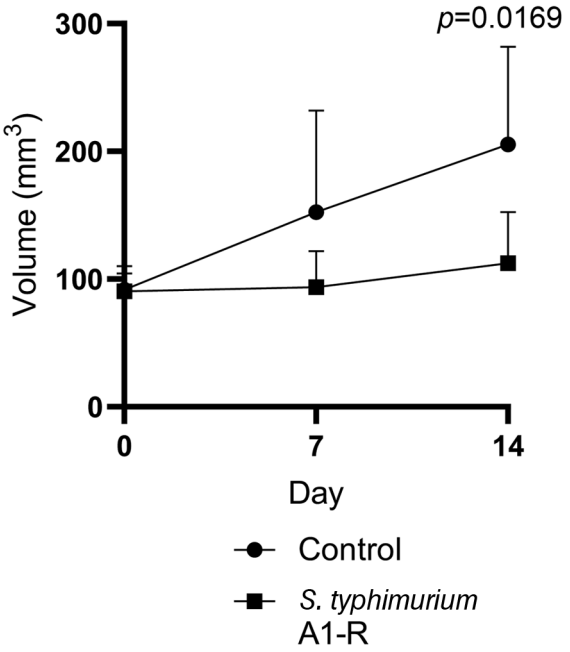

Figure 3. *Efficacy of S. typhimurium A1-R-GFP on the triple-negative matrix-producing breast carcinoma (MPBC) PDOX nude mice. Tumors were measured at the indicated time points after the initiation of treatment. Control group (n=6) vs. S. typhimurium A1-R-GFP-treated group (n=7). Line graphs show the tumor volume at the indicated time points. Error bars represent the mean±SD.*

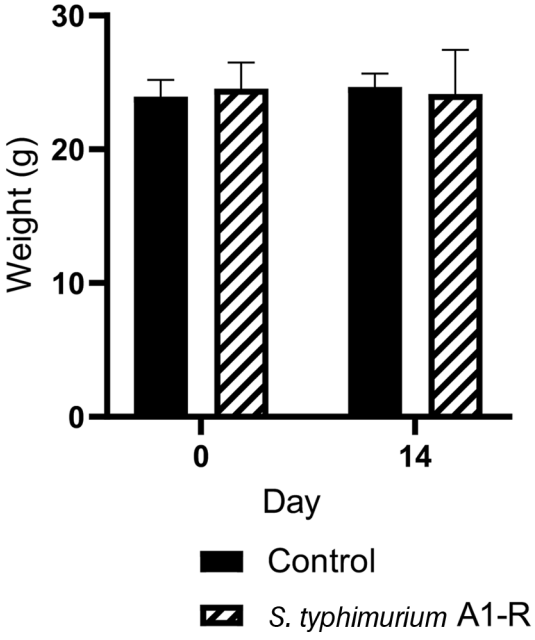

Figure 4. *Effect of S. typhimurium A1-R-GFP on mouse body weight in each group. Bar graphs show the bodyweight of mice at the indicated time points. No significant body weight differences were observed between the groups at each point. Control group (n=6) vs., S. typhimurium A1-R-GFP-treated group (n=7), on day 0, on day 14, p=0.53, 0.73, respectively.*

the rich nutritional milieu of tumors, but it is severely restricted in normal tissue (12). Thus *S. typhimurium* A1-R can directly target tumors as well as serve as an anti-tumor immuno-stimulator. In a previous study, we showed that *S. typhimurium* A1-R enabled CD-8 T-cells to penetrate tumors (33). In the present study, *S. typhimurium* A1-R-GFP primarily localized and proliferated in the triple-negative MPBC tumor and *S. typhimurium* A1-R-GFP was undetectable in the liver and spleen. These results suggest the clinical potential of *S. typhimurium* A1-R for triple-negative MPBC.

Conclusion

S. typhimurium A1-R-GFP exquisitely targeted and arrested the growth of a triple-negative MPBC in a PDOX mouse model without apparent toxicity. The triple-negative MPBC PDOX model allows the development of precise, individualized, improved therapy for patients with this recalcitrant disease.

Conflicts of Interest

AntiCancer Inc. uses PDOX models for contract research. K.H., Y.A., M.Z., N.S., and R.M.H. are or were unsalaried associates of AntiCancer Inc. C.H. is an unsalaried associate of AntiCancer Japan. The Authors declare no competing financial interests.

Authors' Contributions

K.H. and R.M.H designed and performed experiments, analyzed data, and wrote the article; T.M. provided the tumor specimen; C.H. established the patient tumor specimens in nude mice; J.Y., Z.M., N.S., Y.A., and M.B. provided technical support and conceptual advice.: K.H., T.T., and R.M.H. wrote, reviewed, and/or revised the manuscript.

Funding

The present study was funded in part by The Robert M Hoffman Foundation For Cancer Research which had no other role in the study.

Acknowledgements

This paper is dedicated to the memory of A. R. Moossa, M.D., Sun Lee, M.D., Professor Li Jiaxi, and Masaki Kitajima, M.D.

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Received August 1, 2021
Revised August 23, 2021
Accepted August 24, 2021