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Oral-recombinant Methioninase in Combination With Rapamycin Eradicates Osteosarcoma of the Breast in a Patient-derived Orthotopic Xenograft Mouse Model

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Abstract. *Background/Aim: Primary osteosarcoma of the breast is a very rare malignancy that shares histological features with osteosarcoma. It is also highly sensitive to methionine restriction due to methionine addiction. We previously established a patient-derived orthotopic xenograft (PDOX) nude-mouse model derived from tumor tissue of a patient with primary mammary osteosarcoma. In the present study, we investigated the efficacy of oral-recombinant methioninase (o-rMETase), combined with rapamycin, an inhibitor of mammalian target of rapamycin (mTOR) kinase, on a mammary osteosarcoma PDOX nude-mouse model. Materials and Methods: The PDOX mouse model was established by surgically transplanting a specimen of primary osteosarcoma of the breast into the mammary gland of nude mice. Mice implanted with tumors were randomly divided into four groups: Control group, N=5; rapamycin-treated group, N=5; o-rMETase-treated group, N=5; and a group treated with the combination of o-rMETase and rapamycin, N=5. Mice were treated for 2 weeks after transplantation, and tumor volume was measured during the treatment period. Results: Treatment with the combination of rapamycin and o-rMETase eradicated the osteosarcoma of the breast compared to the untreated control (p=0.000008). o-rMETase alone did not significantly inhibit tumor growth, and rapamycin alone only partially inhibited the tumor (p=0.78 and p=0.018, respectively) compared to the untreated control. There was not a significant difference in mouse weight between the groups. Conclusion: The combination of rapamycin and o-rMETase was highly effective against primary osteosarcoma of the breast in a PDOX model, suggesting a future clinical strategy for this rare cancer type that currently has no first-line treatment.*

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Key Words: Breast osteosarcoma, PDOX, nude mice, combination therapy, methioninase, rapamycin, efficacy, synergy, Hoffman effect.

Osteosarcoma arising from the mammary gland is very rare, accounting for 0.2-1% (1, 2) of all breast malignancies. The histology is very similar to that of osteosarcoma arising from bone. There are various theories as to its origin, including mesenchymal-cell origin in the mammary gland, overgrowth of mesenchymal cancer cells in malignant lobular tumors, or overgrowth of the mesenchymal component of metaplastic carcinomas (3, 4). Because osteosarcoma is a very rare cancer, there is currently no first-line treatment.

Recently, an inhibitor of the mammalian target of rapamycin (mTOR) kinase, rapamycin, was reported to be effective against osteosarcoma cell lines (5). Oral-recombinant methioninase (o-rMETase) combined with various chemotherapy was found to be effective against bone osteosarcoma (6). Based on these studies, we examined whether the combination of rapamycin and o-rMETase is effective against a patient-derived orthotopic xenograft (PDOX) nude-mouse model of breast osteosarcoma (7).

Materials and Methods

Mice. Athymic (nu/nu) nude female mice 4 to 6 weeks old (AntiCancer, Inc., San Diego, CA, USA) were used under an AntiCancer, Inc. Institutional. Animal Care and Use Committee

protocol. It was specifically approved for this study and followed the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Animals (Assurance No. A3873-1).

Patient-derived tumor. Surgical specimens of primary osteosarcoma of the mammary gland were previously obtained under protocols approved by the Kaiser Permanente San Diego Medical Center (IRB# 12617).

Establishment of a breast osteosarcoma PDOX nude-mouse model. Tumors from mice subcutaneously implanted with patient-derived osteosarcoma of the breast (Figure 1) were minced to approximately 40 mm^3 and prepared for transplantation. Mice were transplanted with tumor along with normal tissue, using the method of Hozumi (8). For details of these methods, please refer to our previous study (9). Mice were anesthetized with a ketamine mixture. A 1 cm skin incision was made over the left mammary tissue of the nude mice. Using surgical scissors, the skin was peeled away from the dermis to create a pocket (Figure 2A). A fragment of mammary osteosarcoma was inserted into the pocket along with the surrounding normal tissue. The wound was closed with 5-0 PDS-II sutures (Figure 2B).

Treatment scheme. Three weeks after orthotopic transplantation, when tumor volume had reached 70-100 mm^3, treatment was initiated. Each mouse model was randomly assigned to one of four groups of five mice per group as follows: Control phosphate-buffered saline (PBS) (0.2 ml, orally, twice daily); rapamycin (1.0 mg/kg, daily, intraperitoneal injection); o-rMETase (50 units, orally, twice daily); o-rMETase (50 units, orally, twice daily) combined with rapamycin (1.0 mg/kg, daily, intraperitoneal injection) (Figure 3).

Mice were weighed weekly. The short and long axes of the tumors were measured using calipers and tumor volume was determined as $(\text{short axis})^2 \times \text{long axis}/2$. All mice were sacrificed on day 15 after the start of treatment and the tumors were removed for histological evaluation.

Hematoxylin and eosin staining. Procedures for hematoxylin and eosin staining were performed according to standard protocols.

Statistical analyses. A graphical user interface of R (The R Foundation for Statistical Computing, Vienna, Austria), EZR (Saitama Medical Center, Jichi Medical University, Japan), was used to execute all statistical analyses (10). Tukey–Kramer analysis was employed to compare the four groups. Relative tumor volume and relative mouse weight are displayed as the mean with standard deviation. Differences were deemed statistically significant if their probability value was 0.05 or lower.

Results

Treatment efficacy against the breast osteosarcoma PDOX. Figure 4 shows that rapamycin alone significantly inhibited growth of the breast osteosarcoma PDOX compared to the untreated control (p=0.018). o-rMETase alone did not significantly inhibit breast osteosarcoma PDOX growth. The combination of o-rMETase and rapamycin apparently eradicated the breast osteosarcoma PDOX compared to rapamycin alone (p=0.004), o-rMETase alone (p=0.00004) and to the untreated control (p=0.000008).

Figure 1. *Fragmentation of breast osteosarcoma tumor tissue in preparation for transplantation.*

Effect of treatment on body weight. No treatment significantly reduced the body weight of the breast osteosarcoma PDOX mouse model (Figure 5).

Histology of breast osteosarcoma PDOX. o-rMETase alone did not affect the histological phenotype of osteosarcoma PDOX (Figure 6C). Rapamycin alone induced extensive necrosis (Figure 6B). The combined treatment with rapamycin plus o-rMETase apparently eradicated the cancer cells (Figure 6D).

Discussion

Rapamycin has previously demonstrated antitumor efficacy (5). Rapamycin targets the mTOR kinase in the PI3K/AKT/mTOR signalling pathway, which is involved in the regulation of protein translation, cell growth, and autophagy (11).

The present study has shown that rapamycin alone has therapeutic potential in a PDOX mouse model of osteosarcoma of the breast. o-rMETase in combination with rapamycin, was much more effective, eradicating the tumor.

Methionine dependence in cancer cells was first reported by Sugimura *et al.* in 1959 (12). Methionine dependence is due to the methionine addiction of cancer cells, which is called the Hoffman effect (13, 14). Methionine addiction is caused by excessive use of methionine in transmethylation

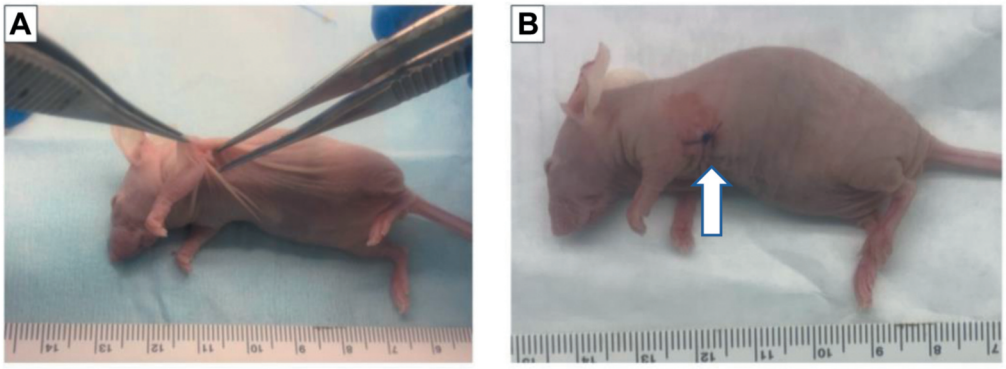

Figure 2. *Patient-derived orthotopic xenograft mouse model of osteosarcoma of the breast. A: Insertion of a fragment of breast osteosarcoma into a pocket on the left of the mammary grand. B: The white arrow indicates where a 5.0 nylon suture was used to close the incision.*

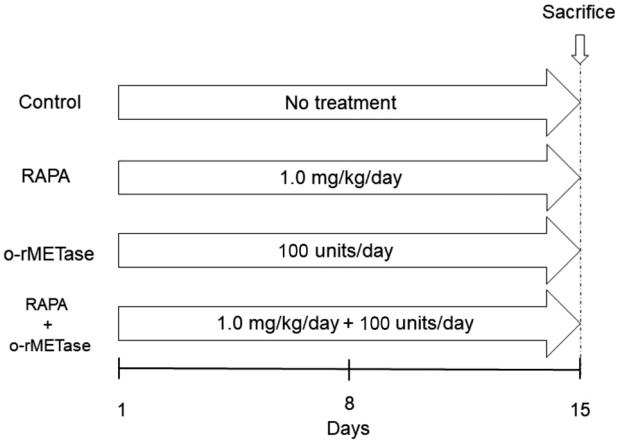

Figure 3. *Treatment scheme for the patient-derived orthotopic xenograft mouse model of osteosarcoma of the breast. RAPA: rapamycin; o-rMETase: oral recombinant methioninase.*

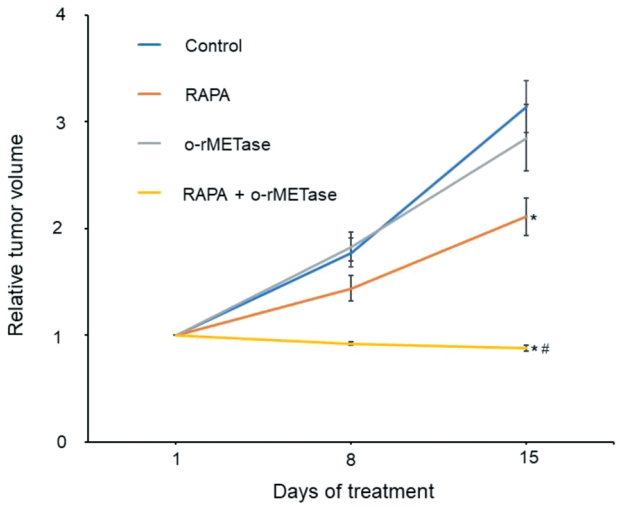

Figure 4. *Efficacy of treatment on patient-derived breast osteosarcoma orthotopic xenograft. Line graphs show tumor volume at the indicated times relative to that at the start of treatment. Significantly different at $p<0.05$ from: *control and #single treatments. Data are the mean±standard error. RAPA: rapamycin; o-rMETase: oral recombinant methioninase.*

reactions that deplete endogenous free methionine and S-adenosylmethionine under methionine limitation (15-17).

Our laboratory has focused on the use of o-rMETase to treat malignant tumors by methionine restriction. Previous reports have shown that methionine restriction selectively traps cancer cells in the S/G_2 phase of the cell cycle, where they are sensitive to most cytotoxic chemotherapy and can be successfully eradicated (18, 19).

The combination of rapamycin and o-rMETase was shown in the present study to be highly effective for osteosarcoma of the breast and should be tested clinically against mammary osteosarcoma a rare cancer that does not have first-line therapy.

rMETase alone or at combination with chemotherapy has been shown to be active clinically with major cancer types (20-24). Synergy of methionine restriction and chemotherapy was discovered by our team (25) and is called the Hoffman protocol, which targets methionine addiction which is the fundamental chracteristic of cancer (13, 26-28).

Conflicts of Interest

The Authors declare that they have no conflicts of interest in relation to this study. AntiCancer Inc. uses PDOX models for contract research.

Authors' Contributions

N.M., C.S. and N.F.W. conceived the study, N.M., C.S., C.H., K.O., Y.K., and Y.A. performed the experiments and Q.H. and R.M.H. provided scientific advice. N.M. wrote the paper and R.M.H. revised the paper.

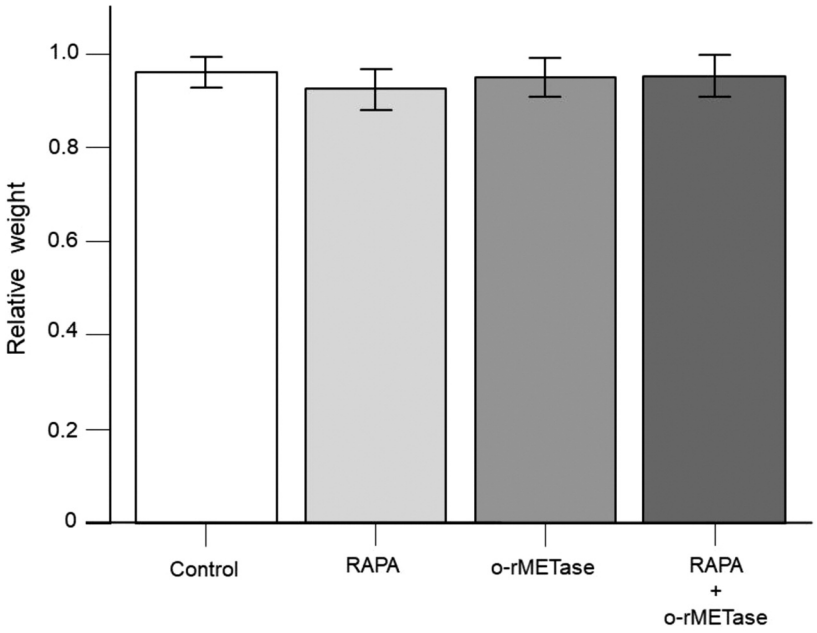

Figure 5. *Mouse body weight. Bar graphs show the body weight of mice from each group at day 15 relative to day 1 of treatment. Data are the mean±standard error: RAPA: rapamycin; o-rMETase: oral recombinant methioninase.*

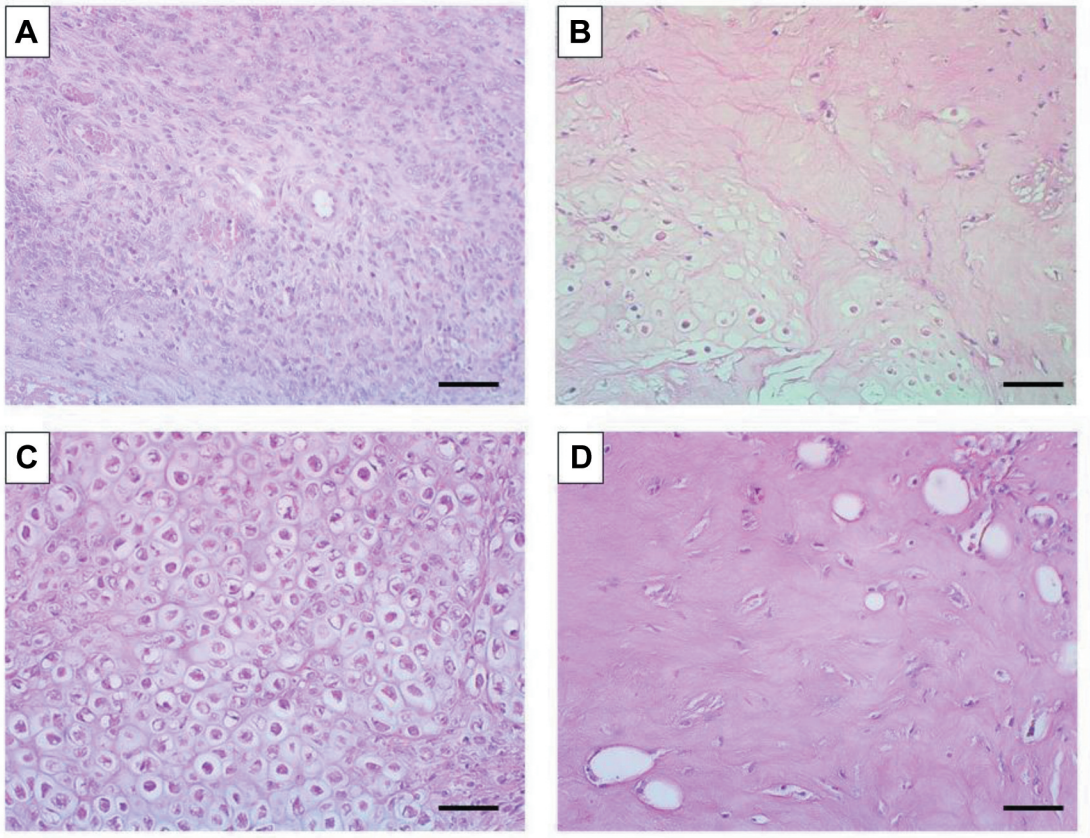

Figure 6. *Representative photomicrographs of hematoxylin and eosin-stained sections of tissue from patient-derived breast osteosarcoma orthotopic xenograft (PDOX) nude-mouse models in the untreated control (A); rapamycin-treated (B); oral recombinant methioninase-treated (C); and combination-treated (rapamycin with oral recombinant methioninase) (D) Magnification: 200×. Scale bar: 50 μm.*

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