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Efficacy of Recombinant Methioninase on Late-stage Patient Cancer in the Histoculture Drug Response Assay (HDRA) as a Potential Functional Biomarker of Sensitivity to Methionine-restriction Therapy in the Clinic

YUTARO KUBOTA[1,2,3], MASATO SASAKI[4], QINGHONG HAN[1], CHIHIRO HOZUMI[5], TAKUYA TSUNODA[3] 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]*Division of Internal Medicine, Department of Medical Oncology, Showa University School of Medicine, Tokyo, Japan;*
[4]*University of Fukui Faculty of Medical Sciences, Department of Thoracic Surgery, Fukui, Japan;*
[5]*AntiCancer Japan Inc., Narita, Japan*

Abstract. *Background/Aim: The present study utilized the three-dimensional histoculture drug response assay (HDRA) to determine the efficacy of recombinant methioninase (rMETase) on tumor tissue resected from patients with late-stage cancer, as a functional biomarker of sensitivity to methionine restriction therapy. Patients and Methods: Resected peritoneal-metastatic cancer, including colorectal cancer, pancreatic cancer, ovarian cancer, and pseudomyxoma were placed on Gelform in RPMI 1640 medium for seven days and treated with rMETase from 2.5 U/ml to 20 U/ml. Cell viability was determined using the MTT assay. A total of 48 patients with late-stage cancer underwent testing for rMETase responsiveness using the HDRA. Results: Colorectal cancer and pseudomyxoma had the highest sensitivity to rMETase. Pancreatic and ovarian cancer also responded to rMETase, but to a lesser degree. Conclusion: Patients with tumors with at least 40% sensitivity to rMETase in the HDRA are being considered as candidates for methionine restriction therapy, which includes the use of rMETase in combination with a low-methionine diet.*

Correspondence to: Robert M. Hoffman, Ph.D., AntiCancer Inc, 7917 Ostrow St, Suite B, San Diego, CA, 92111, U.S.A. Tel: +1 6198852284, Fax: +1 6198852284, e-mail: all@anticancer.com

Key Words: Colorectal cancer, appendix cancer, pancreatic cancer, ovarian cancer, pseudomyxoma, histoculture drug response assay (HDRA), methioninase, methionine restriction, efficacy, biomarker.

Methionine addiction is a fundamental and universal hallmark of cancer (1-9) termed the Hoffman effect (10). Numerous pre-clinical studies, both *in vitro* and in mouse models of cancer (11), have shown that all tested cancers are methionine addicted and respond to methionine restriction with recombinant methioninase (rMETase). Recently, clinical case studies targeting methionine addiction with oral rMETase (o-rMETase) have shown potential for efficacy against recalcitrant cancer (12-17). It is therefore important to identify patients who would be appropriate candidates for o-rMETase treatment.

We developed the histoculture drug response assay (HDRA) almost forty years ago (18-22). The HDRA uses 3-dimensional cultures of tumor fragments on Gelfoam substrates. There is a high correlation of HDRA results and chemotherapy outcome in the clinic, including survival (20-22).

In the present study, we utilized the HDRA to determine sensitivity of highly recalcitrant peritoneal-metastatic cancer to rMETase. We propose that rMETase sensitivity in the HDRA is a potential biomarker to indicate methionine-restriction therapy in the clinic.

Patients and Methods

Patients. In the present study, we focused on highly recalcitrant peritoneal-metastatic cancers. Tissue samples were collected from 27 colorectal cancer cases, an appendix cancer case, six pancreatic cancer cases, eight ovarian cancer cases, and seven pseudomyxoma

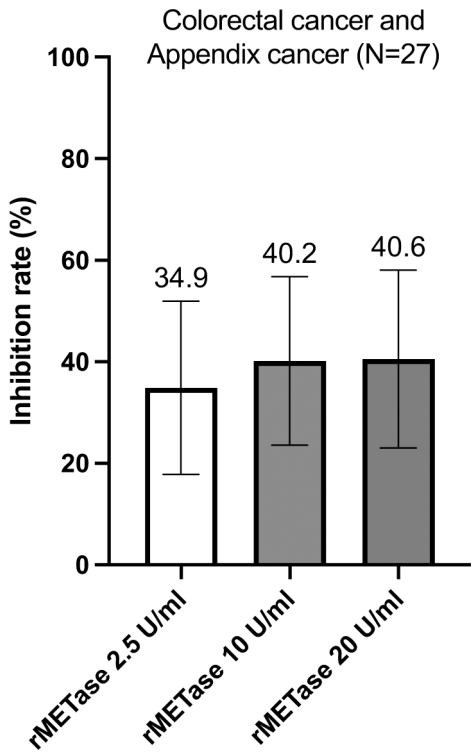

Figure 1. *Histoculture drug response assay (HDRA) results of the sensitivity of peritoneal-metastatic colorectal cancer and appendix cancer to recombinant methioninase (rMETase) (N=27).*

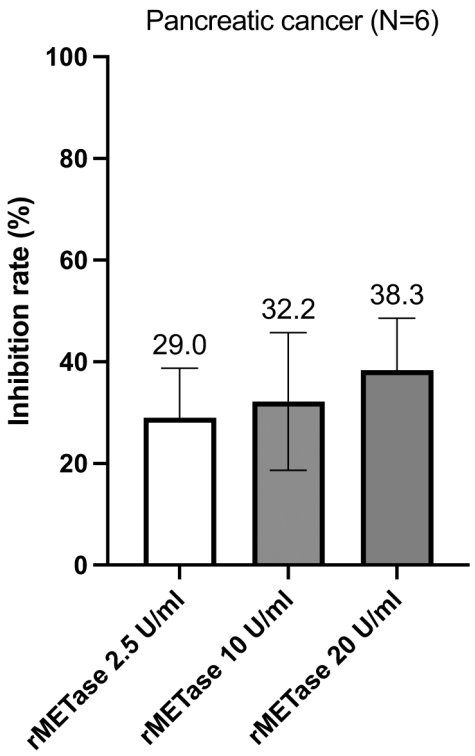

Figure 2. *Histoculture drug response assay (HDRA) results of the sensitivity of peritoneal-metastatic pancreatic cancer to recombinant methioninase (rMETase) (N=6).*

patients who underwent surgery at the Kishiwada Tokushukai Hospital in Japan. The experimental use of the chemosensitivity test was approved by the Institutional Research Committee in Kishiwada Tokushukai Hospital. HDRA testing was approved by the Institutional Review Board for the University of Fukui Hospital. All patients were informed of the nature of this study, and written informed consent was obtained.

HDRA. The HDRA was performed as an *in vitro* assay for assessing rMETase sensitivity, as described in prior studies (18-22). The collagen sponge gels (Gelfoam®) used in this study were obtained from Pfizer Japan Inc. (Tokyo, Japan). Gelfoam was manufactured using pig skin as the primary raw material. The cancerous portions of the specimens were cut into fragments weighing approximately 10 mg. These fragments were placed on the surface of Gelfoam in 24-well microplates. The plates were incubated for seven days at 37˚C with a humidity level of 100% and atmospheric composition of 95% air and 5% CO_2 in the presence of rMETase dissolved in RPMI 1640 medium containing 20% fetal calf serum. rMETase was tested at 2.5 U/ml, 10 U/ml, and 20 U/ml. The incubation temperature was maintained at 37˚C. Following specimen histoculture, a solution consisting of 100 µl Hank's balanced salt solution containing 0.1 mg/ml type I collagenase (Sigma, St. Louis, MO, USA) and 100 µl of a solution of 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dissolved in phosphate-buffered saline (9.6 mg/ml) was added to each culture well. The specimens were then incubated for an additional 16 h. After an extraction process using dimethylsulfoxide, the absorbance of the solution in each well was measured at a wavelength of 540 nm (with control at 630 nm) using a microplate reader (Spectra Max M5; Molecular Devices LLC, San Jose, CA, USA). The absorbance per mg of cultivated tumor tissue was determined by averaging the absorbance values (OD) obtained from three separate culture wells. The weight of the tumor tissue was measured before initiating the culture. The calculation of the inhibition rate was performed using the following formula:

Inhibition rate (%)=(1 - mean OD/well of treated well/mean OD/well of control well) ×100

rMETase production and formulation. *Escherichia coli* that had been transformed with the *methioninase* gene derived from *Pseudomonas putida* was fermented in order to produce rMETase. The purification steps comprised heating at 60˚C, followed by polyethylene glycol precipitation and diethylaminoethyl-sepharose fast-flow non-exchange chromatography. rMETase was dissolved in a 5 mg/ml solution of pyridoxal 5'-phosphate (PLP) in normal saline (23, 24).

Statistics. Statistical analyses were performed using GraphPad Prism 9.4.0 (GraphPad Software, Inc., San Diego, CA, USA). The data are presented as the mean and standard deviation. The significance threshold was less than or equal to 0.05.

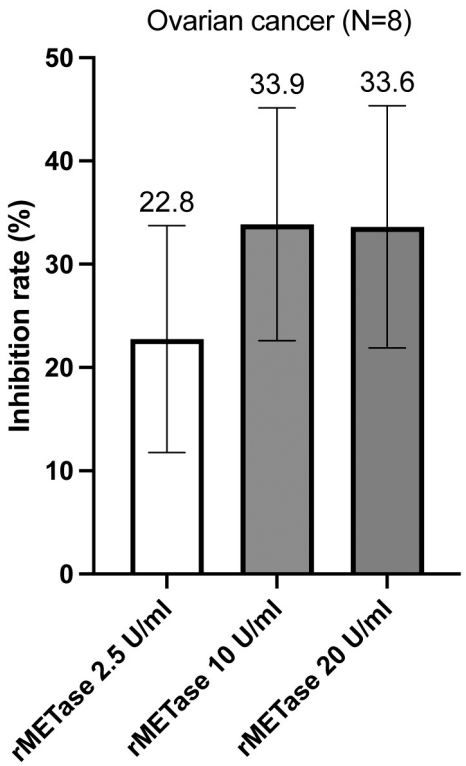

Figure 3. *Histoculture drug response assay (HDRA) results of the sensitivity of peritoneal-metastatic ovarian cancer to recombinant methioninase (rMETase) (N=8).*

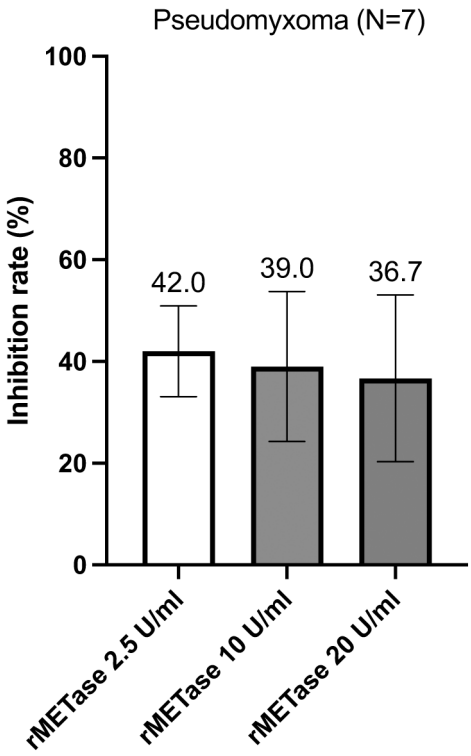

Figure 4. *Histoculture drug response assay (HDRA) results of the sensitivity of peritoneal-metastatic pseudomyxoma to recombinant methioninase (rMETase) (N=7).*

Results

Sensitivity of peritoneal-metastatic colorectal cancer and appendix cancer to rMETase in the HDRA. Peritoneal metastatic colorectal and appendix cancer (N=27) were sensitive to rMETase in the HDRA with an inhibition rate ranging from 34.9 % to 40.6 % (Figure 1). There was no dose-response effect as 2.5 units rMETase was almost as effective as 10 and 20 units/ml rMETase.

Sensitivity of peritoneal-metastatic pancreatic cancer to rMETase in the HDRA. Peritoneal-metastatic pancreatic cancer (N=6) was sensitive to rMETase in the HDRA with an inhibition rate ranging from 29.0 to 38.3% with moderate dose-response to rMETase from 2.5 to 20 units/ml (Figure 2).

Sensitivity of peritoneal-metastatic ovarian cancer to rMETase in the HDRA. Peritoneal-metastatic ovarian cancer (N=8) was sensitive to rMETase in the HDRA with an inhibition rate ranging from 22.8 to 33.9 % for 2.5 units/ml to 20 units/ml rMETase (Figure 3).

Sensitivity of peritoneal-metastatic pseudomyxoma to rMETase in the HDRA. Peritoneal-metastatic pseudomyxoma (N=7) was sensitive to rMETase in the HDRA with an inhibition rate ranging from 36.7 to 42.0 %. Dose response was not observed for rMETase from 2.5 to 20 units/ml (Figure 4).

Discussion

Methionine addiction is a universal hallmark of cancer (1-9). Therefore, it is a prime therapeutic target, especially for highly-recalcitrant cancer such as peritoneal-metastatic cancer, described in the present report. The present study determined the sensitivity of peritoneal-metastatic cancer to rMETase in the HDRA. The HDRA allows tissues to take their natural shape in 3-dimensions on a flexible *in vivo*-like substrate, enabling clinically-accurate drug sensitivity results to be obtained (18-22).

Colorectal cancer, appendix cancer, and pseudomyxoma tended to be more sensitive to rMETase than ovarian and pancreatic cancer in the HDRA. In pancreatic and ovarian cancer, there was moderate dose-dependency of rMETase in the HDRA.

When cancer has spread to the peritoneum, the disease is highly recalcitrant and most often lethal. The present study shows for the first time the response of peritoneal-metastatic cancer to rMETase. Since the HDRA has previously shown a high correlation with clinical results (20, 22), the present

results suggest that rMETase has clinical potential as therapy of peritoneal-metastatic cancer. The present study suggests that sensitivity of a cancer to rMETase in the HDRA could be a functional biomarker for sensitivity to methionine-restriction therapy in the clinic.

Conflicts of Interest

The Authors declare no competing interests regarding this work.

Authors' Contributions

MS performed experiments. YK, and RMH wrote the article. QH provided methioninase. CH and TT gave critical suggestions.

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