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Research Paper

Intra-tumor L-methionine level highly correlates with tumor size in both pancreatic cancer and melanoma patient-derived orthotopic xenograft (PDOX) nude-mouse models

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ABSTRACT

An excessive requirement for methionine (MET) for growth, termed MET dependence, appears to be a general metabolic defect in cancer. We have previously shown that cancer-cell growth can be selectively arrested by MET restriction such as with recombinant methioninase (rMETase). In the present study, we utilized patient-derived orthotopic xenograft (PDOX) nude mouse models with pancreatic cancer or melanoma to determine the relationship between intra-tumor MET level and tumor size. After the tumors grew to 100 mm³, the PDOX nude mice were divided into two groups: untreated control and treated with rMETase (100 units, i.p., 14 consecutive days). On day 14 from initiation of treatment, intra-tumor MET levels were measured and found to highly correlate with tumor volume, both in the pancreatic cancer PDOX ($p<0.0001$, $R^2=0.89016$) and melanoma PDOX ($p<0.0001$, $R^2=0.88114$). Tumors with low concentration of MET were smaller. The present results demonstrates that patient tumors are highly dependent on MET for growth and that rMETase effectively lowers tumor MET.

INTRODUCTION

Cancer cells have an elevated requirement for methionine (MET) compared to normal cells. This phenomena is termed MET dependence [1]. MET restriction arrests tumor growth and induces a selective S/G_2-phase cell-cycle arrest of cancer cells *in vitro* and *in vivo* [2–5].

MET dependence appears to be due to excess use of MET for aberrant transmethylation reactions, termed the Hoffman effect [6–11], analogous to the Warburg effect for glucose in cancer [12]. The excessive and aberrant use of MET in cancer is observed in [^{11}C] MET PET imaging, where high uptake of [^{11}C] MET results in a very strong and selective tumor signal compared with normal tissue background. [^{11}C] MET is superior to

[^{18}C] fluorodeoxyglucose (FDG)- PET for PET imaging, suggesting MET dependence is more tumor-specific than glucose dependence [13–15].

A purified MET cleaving enzyme, methioninase (METase), from *Pseudomonas putida* has been found previously to be an effective antitumor agent *in vitro* as well as *in vivo* [16–19]. For the large-scale production of METase, the gene from *P. putida* has been cloned in *Escherichia coli* and a purification protocol for recombinant METase (rMETase) has been established with high purity and low endotoxin [20–25].

We previously reported on the efficacy of rMETase against a BRAF-V600E mutant melanoma patient-derived orthotopic xenograft (PDOX) nude mouse model and that rMETase sensitized the melanoma PDOX to temozolomide (TEM) [26].

In the present study, we used PDOX nude mouse models with pancreatic cancer and melanoma to demonstrate the relationship between intra-tumor MET level and tumor size, using rMETase to lower tumor MET.

RESULTS AND DISCUSSION

Intra-tumor MET levels highly correlated with tumor volume in both the pancreatic cancer (p<0.0001, R^2=0.89016) (Figure 1) and melanoma PDOX models (p<0.0001, R^2=0.88114) (Figure 2). Tumors with low concentration of MET were smaller in size. Tumors treated with rMETase had lower concentration of MET and were smaller in size than untreated tumors (Table 1).

The present study shows a direct relationship between the intra-tumor MET level and tumor size using PDOX models of pancreatic cancer and melanoma, further demonstrating the MET dependence of cancer, in this case, using patient tumors.

The excessive requirement for MET termed MET dependence appears to be a general metabolic defect in cancer. Sugimura *et al.* showed that rat tumor growth was slowed by giving the rats a defined diet depleted in MET [27]. It was observed that L5178Y mouse leukemia cells in culture required very high levels of MET to proliferate [28]. Subsequently, most cancer cell lines were found to be MET dependent [29, 30]. These cell lines were derived from multiple cancer types including liver, ovarian, submaxillary, brain, lung, bladder, prostate, breast, kidney, cervical, colon, fibrosarcoma, osteosarcoma, rhabdomyosarcoma, leiomyosarcoma, neuroblastoma, glioblastoma, pancreatic and melanoma. The occurrence of MET dependence among these diverse cancer types suggests that methionine dependence is a general phenomenon

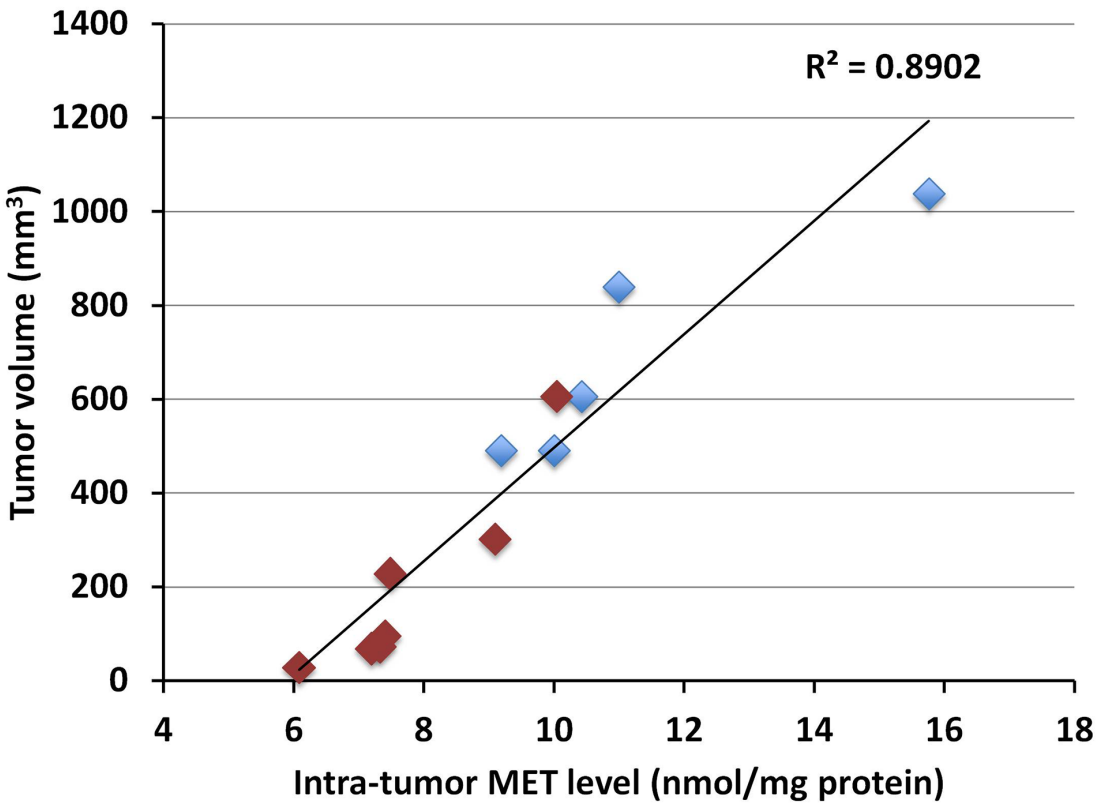

Figure 1: Correlation between tumor volume and intra-tumor MET level in the pancreatic cancer PDOX. Blue box: untreated controls; Red box: treated with rMETase. Please see the Materials and Methods for details.

Table 1: Intra-tumor MET levels (nmol/mg protein) and volume (mm^3) after rMETase treatment

MET concentration			
	Untreated control	**Treated with rMETase**	***p*-value**
Pancreatic cancer PDOX	11.3 ± 0.87	7.80 ± 0.73	$p = 0.0006$
Melanoma PDOX	8.88 ± 1.05	3.65 ± 0.57	$p = 0.0003$
Tumor volume			
	Untreated control	**Treated with rMETase**	***p*-value**
Pancreatic cancer PDOX	693.9 ± 239.6	200.5 ± 204.2	$p = 0.0032$
Melanoma PDOX	3755.2 ± 484.3	857.9 ± 262.6	$p < 0.0001$

in cancer. The present results further substantiate this assumption.

Human patient tumors, including tumors of the colon, breast, ovary, prostate, and melanoma, were previously found to be MET dependent in Gelfoam® histoculture [31]. Mouse models of human cell lines were previously shown to be inhibited by rMETase [32–34].

PDOX models of Ewing's sarcoma [35] and melanoma [26] were also shown to be MET dependent and inhibited by rMETase.

This is the first report that intra-tumor MET levels highly correlated with tumor volume. These results demonstrate that MET restriction, using rMETase, has promising clinical potential.

Previously-developed concepts and strategies of highly-selective tumor targeting can take advantage of molecular targeting of tumors, including tissue-selective therapy which focuses on unique differences between normal and tumor tissues [36–41].

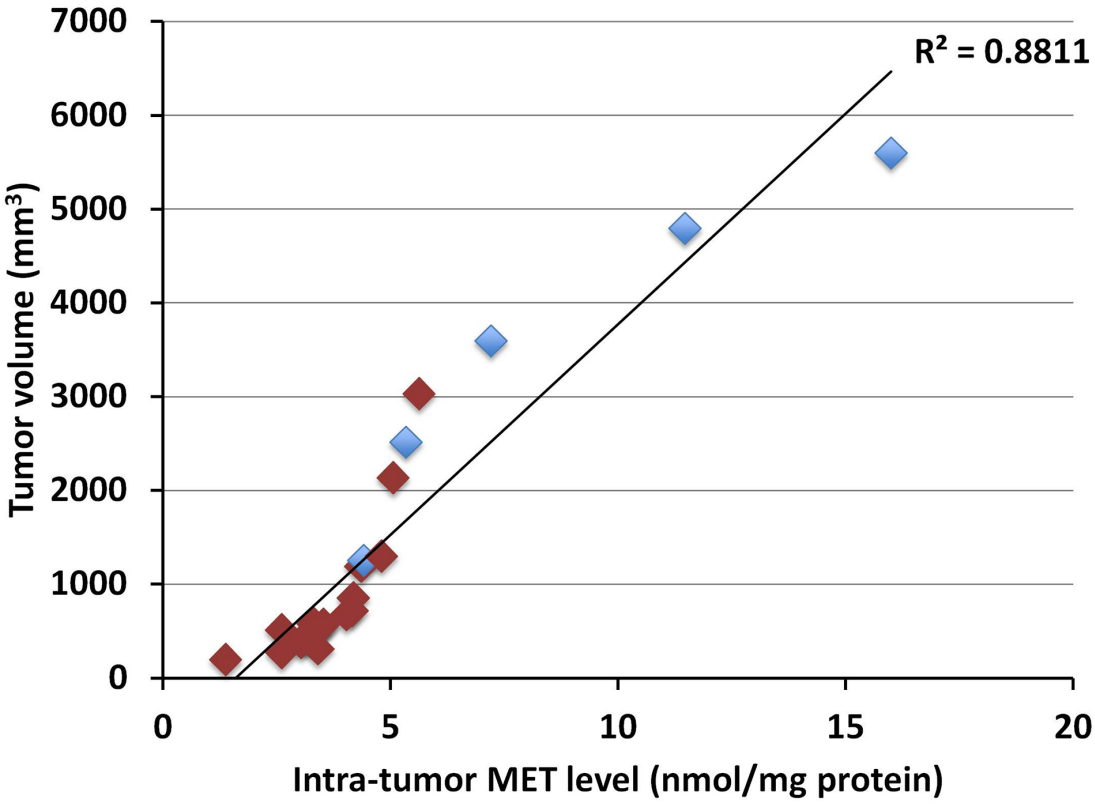

Figure 2: Correlation between tumor volume and intra-tumor MET level in the melanoma PDOX. Blue box: untreated controls; Red box: treated with rMETase. Please see the Materials and Methods for details.

MATERIALS AND METHODS

Mice

Athymic *nu/nu* nude mice (AntiCancer Inc., San Diego, CA), 4–6 weeks old, were used in this study. Mice were housed in a barrier facility on a high efficacy particulate arrestance (HEPA)-filtered rack under standard conditions of 12-hour light/dark cycles. The animals were fed an autoclaved laboratory rodent diet. All animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Animals under Assurance Number A3873-1. All mouse surgical procedures and imaging were performed with the animals anesthetized by subcutaneous injection of a ketamine mixture (0.02 ml solution of 20 mg/kg ketamine, 15.2 mg/kg xylazine, and 0.48 mg/kg acepromazine maleate). The response of animals during surgery was monitored to ensure adequate depth of anesthesia. The animals were observed on a daily basis and humanely sacrificed by CO_2 inhalation if they met the following humane-endpoint criteria: severe tumor burden (more than 20 mm in diameter), prostration, significant body weight loss, difficulty breathing, rotational motion and body temperature drop [26].

Patient-derived pancreatic cancer

The pancreatic tumor was established in nude mice at the MD Anderson Cancer Center under IRB approval and written informed patient consent [42–49].

Surgical orthotopic implantation (SOI) of pancreatic cancer

For the pancreatic cancer PDOX, tumor fragments (5 mm^3) were initially implanted subcutaneously in nude mice. After five weeks, the subcutaneously-implanted tumors grew to more than 10 mm in diameter. The subcutaneously-grown tumors were then harvested and cut into small fragments (3 mm^3). After nude mice were anesthetized with the ketamine solution described above, a 1-2 cm skin incision was made on the left side abdomen through the skin, fascia and peritoneum and pancreas was exposed. Surgical sutures (8-0 nylon) were used to implant tumor fragments onto the tail of pancreas to establish the PDOX model. The wound was closed with a 6-0 nylon suture (Ethilon, Ethicon, Inc., NJ, USA) [50, 51].

Patient-derived melanoma

The melanoma patient PDOX was previously established from a patient diagnosed with a melanoma of the right chest wall under UCLA IRB approval and written informed patient consent [26, 52–55].

SOI of melanoma

After subcutaneously-implanted tumors grew to more than 10 mm in diameter, the subcutaneously-grown tumors were then harvested and cut into small fragments (3 mm^3). After nude mice were anesthetized with the ketamine solution described above, a 5-mm skin incision was made on the right chest into the chest wall in order to match the patient, which was split to make space for the melanoma tissue fragment. A single tumor fragment was implanted orthotopically into the space to establish the PDOX model. The wound was closed with a 6-0 nylon suture (Ethilon, Ethicon, Inc., NJ, USA) [26, 52–55].

Recombinant methionase (rMETase) production

Recombinant L-methionine α-deamino-γ-mercaptomethane lyase (recombinant methioninase [rMETase]) [EC 4.4.1.11] from *Pseudomonas putida* has been previously cloned and was produced in *Escherichia coli* (AntiCancer, Inc., San Diego, CA) [20, 23]. rMETase is a homotetrameric PLP enzyme of 172-kDa molecular mass [20, 25].

Treatment study design

PDOX mouse models were randomized into two groups: untreated control; rMETase (100 units, i.p., 14 consecutive days). Tumor length and width were measured at post-treatment. Tumor volume was calculated with the following formula: Tumor volume (mm^3) = length (mm) × width (mm) × width (mm) × ½ [26].

Intra-tumor MET level analysis

Each tumor was sonicated for 30 seconds on ice and centrifuged at 12,000 rpm for 10 minutes. Supernatants were collected and protein levels were measured using the Coomassie Protein Assay Kit (Thermo Scientific, Rockford, IL). Protein levels were calculated from a standard curve obtained with a protein standard, bovine serum albumin (BSA). MET levels were determined with the HPLC procedure described previously. MET levels were calculated per mg tumor protein [26].

Statistical analysis

JMP version 11.0 was used for analysis of variance (ANOVA). A probability value of $P \leq 0.05$ was considered statistically significant.

CONCLUSIONS

Currently melanoma [56–59] and pancreatic cancer [60, 61] are recalcitrant diseases with no reliable therapy. The results of the present study indicate that rMETase has

general clinical potential to improve the outcome for both diseases as non-BRAF-V600E melanoma is also sensitive to rMETase [62].

DEDICATION

This paper is dedicated to the memory of A. R. Moossa, M.D., Sun Lee, M.D. and Shigeo Yagi, Ph.D.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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