Biochemical and Biophysical Research Communications 518 (2019) 306–310

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Efficacy of oral recombinant methioninase combined with oxaliplatinum and 5-fluorouracil on primary colon cancer in a patient-derived orthotopic xenograft mouse model

Jun Ho Park [a, b, c], Ming Zhao [a], Qinghong Han [a], Yu Sun [a, b], Takashi Higuchi [a, b], Norihiko Sugisawa [a, b], Jun Yamamoto [a, b], Shree Ram Singh [d, *], Bryan Clary [b], Michael Bouvet [b], Robert M. Hoffman [a, b, **]

[a] AntiCancer Inc, San Diego, CA, USA
[b] Department of Surgery, University of California, San Diego, CA, USA
[c] Department of Surgery, Kangdong Sacred Heart Hospital, Hallym University College of Medicine, Seoul, South Korea
[d] Basic Research Laboratory, National Cancer Institute, Frederick, MD, USA

ARTICLE INFO

Article history:
Received 30 July 2019
Accepted 8 August 2019
Available online 14 August 2019

Keywords:
Colon cancer
Primary tumor
Red fluorescent protein
Patient-derived orthotopic xenograft
Methioninase

ABSTRACT

The aim of this study was to determine the efficacy of oral recombinant methioninase (*o*-rMETase) on a colon cancer primary tumor using a patient-derived orthotopic xenograft (PDOX) nude mouse model. Forty colon cancer primary tumor PDOX mouse models were divided into 4 groups of 10 mice each (total 40 mice) by measuring the tumor size. The groups were as follows: untreated control; 5-fluorouracil (5-FU) (50 mg/kg, once a week for two weeks, N = 10 mice) and oxaliplatinum (OXA) (6 mg/kg, once a week for two weeks, N = 10 mice); *o*-rMETase (100 units/day, oral 14 consecutive days, N = 10 mice); combination of 5-FU + OXA and *o*-rMETase (N = 10 mice). All treatments inhibited tumor growth compared to the untreated control. The combination of 5-FU + OXA and *o*-rMETase was significantly more efficacious than other treatments. The present study demonstrates the efficacy of *o*-rMETase combination therapy on a PDOX colon cancer primary tumor, suggesting potential clinical development of *o*-rMETase in recalcitrant cancer.

Published by Elsevier Inc.

1. Introduction

Colorectal cancer is the third most common cancer diagnosed worldwide. The American Cancer Society's estimates that 145,600 new cases of colorectal cancer and 51,020 people will die from this disease in 2019 [1]. By 2030, it is estimated that globally more than 2.2 million new cases and 1.1 million deaths will occur because of colorectal cancer. Most of the primary cancers arising in the colon are adenocarcinomas. Current treatment strategies for colon cancer mainly includes surgical resection, adjuvant chemotherapy and immunotherapy.

The elevated methionine (MET) requirement of cancer cells is referred to as MET dependence or MET addiction [1] and appears due to elevated use of MET for transmethylation reactions [2–5]. The elevated MET use in cancer MET-dependence/addiction is called the “Hoffman effect” analogous to the Warburg effect of excess glucose utilization by cancer cells [6]. Comparison of radioactive MET and radioactive glucose PET imaging has shown a stronger signal with MET [7] suggesting that the Hoffman effect is more pronounced than the Warburg effect and maybe the most important hallmark of cancer [8,9].

MET restriction (MR) by recombinant methioninase (rMETase) targets MET-dependence/addiction of cancer and can inhibit the growth of cancer cells in vitro and in vivo [8]. rMETase has been used as a treatment strategy for various types of cancer [10–16].

Previous studies have shown that intra-peritoneal rMETase injection (ip-rMETase) was effective against patient-derived orthotopic xenograft (PDOX) mouse models of recalcitrant cancer [17–21]. Recently, we reported that oral recombinant methioninase

* Corresponding author. Basic Research Laboratory, National Cancer Institute, Frederick, MD, 21702, USA.
** Corresponding author. AntiCancer, Inc, 7917 Ostrow St, San Diego, CA, 92111, USA.
E-mail addresses: singhshr@mail.nih.gov (S.R. Singh), all@anticancer.com (R.M. Hoffman).

https://doi.org/10.1016/j.bbrc.2019.08.051
0006-291X/Published by Elsevier Inc.

(*o*-rMETase) was significantly more effective than intraperitoneal injection rMETase (ip-rMETase) indicating the potential widespread use of rMETase for cancer treatment [22–24]. However, in orthotopic models, it is difficult to visualize tumor growth and metastasis. To address this problem of imaging such orthotopic tumor grafts, we have recently developed the technology to introduce fluorescent protein-expressing stroma into tumors by passaging tumor grafts through transgenic nude mice expressing fluorescent proteins [25].

The present report demonstrates the efficacy of *o*-rMETase using a PDOX primary colon cancer nude mouse model with brightly labeled red fluorescent protein (RFP)-expressing stroma for imaging in a PDOX model.

2. Materials and methods

2.1. Mice

Four to six-week old athymic *nu/nu* nude mice and transgenic RFP expressing athymic nu/nu mice were obtained from AntiCancer Inc. (San Diego, CA). All surgical procedures and imaging were performed in accordance with an AntiCancer Institutional Animal Care and Use Committee (IACUC)-protocol specifically approved for this study, and 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. Mouse housing, feeding, surgical process, and imaging were conducted, and mice were humanely sacrificed as previously described [35].

2.2. Patient-derived tumor

The primary tumor was resected from a patient diagnosed with colon cancer at the Division of Surgical Oncology, University of California, San Diego (UCSD). A fresh sample of colon cancer was obtained immediately after patient surgery with informed patient consent and Institutional Review Board (IRB) approval. The tumor was cut into fragments and initially implanted subcutaneously in nude mice. The subsequent subcutaneous tumor was harvested and used for orthotopic implantation. We have previously reported the establishment of an orthotopic model of patient-derived colon cancer with the technique of surgical orthotopic implantation (SOI), which was used in the present study [36].

2.3. Establishment of a PDOX mode of colon cancer with red fluorescent stroma

Patient colon-cancer tumors growing in nude mice were harvested, cut into 5 mm fragments, and implanted subcutaneously in transgenic RFP-expressing nude mice (Fig. 1A). After two passages in RFP-expressing nude mice, tumors stably containing RFP-expressing stromal cells were obtained and cut into fragments. After non-transgenic nude mice were anesthetized with ketamine, 1–2 cm skin incisions were made at the midline of the abdomen. Surgical sutures (8-0 nylon) were used to implant tumor fragments onto the cecum. Wounds were closed using 6-0 nylon sutures [36].

2.4. Production of rMETase

rMETase is a homotetrameric PLP enzyme of 172-kDa molecular mass. Production of rMETase has been described [37].

2.5. Treatment study design in the PDOX model of colon cancer

Four weeks after surgical orthotopic implantation of colon cancer RFP tumors, non-invasive external red fluorescent imaging was performed in all mice (total 40 mice) and they were divided into 4 groups (10 mice/per treatment group) by measuring the tumor size and fluorescence intensity.

The first group served as a negative control and did not receive treatment (N = 10 mice). Mice in the second group were treated once a week for two weeks with intraperitoneal injection of 50 mg/kg 5-FU, and 6 mg/kg OXA (N = 10 mice). Mice in the third group received 100 units/day of *o*-rMETase by gavage for 2 weeks (N = 10 mice). Mice in the fourth group received the combination of all 3 drugs (N = 10 mice).

2.6. Fluorescence imaging of colon cancer and measurement of tumor weight ad volume

Four weeks after implantation, mice were anesthetized for measurement of tumor size and non-invasive external red fluorescence imaging. External red fluorescence images were obtained twice a week. Fluorescence intensity were measured and calculated using the UVP iBox® (Analytik Jena, Germany) and FluoroVivo (INDEC System Inc., Santa Clara, CA). Six weeks after RFP-expressing tumors were implanted, mice were then sacrificed for direct measurements of tumor weight and volume. Frozen tissue sections were observed for fluorescence with an FV1000 confocal laser microscope (Olympus Corp, Tokyo Japan). Excitation wavelength for RFP fluorescence was 559 nm. Tissues were viewed under 10X and 60X objective lenses.

2.7. Intra-tumor L-MET level analysis

Each tumor was sonicated for 30 s on ice and centrifuged at 12,000 rpm for 10 min. Supernatants were collected, and protein concentration was measured using the Coomassie Protein Assay Kit (Thermo Scientific, Rockford, IL) Protein concentrations were calculated form a standard curve obtained with a protein standard, bovine serum albumin (BSA). L-MET levels were determined with the high-performance liquid chromatography (HPLC) procedure described previously [38,39]. Standardized L-MET levels were calculated per mg tumor protein.

2.8. Statistical analysis

Differences in the weight and volume of the tumors between the groups were assessed for significance using an independent-samples *t* tests. Pearson correlation coefficient and linear regression were used to assess the various possible relationships among different variables. *p*-values of less than 0.05 was considered statistically significant.

3. Results

3.1. Non-invasive RFP images of the PDOX primary colon cancer model

Fragments of a patient colon tumor were implanted in the cecum of non-transgenic nude mice. These tumors contained red fluorescent stroma from previous growth in RFP transgenic nude mice. Non-invasive external fluorescence images and intravital images via laparotomy of the RFP-expressing tumors were obtained (Fig. 1B). Fluorescence intensity of the tumor visualized with laparotomy had a strong statistical correlation with tumor volume ($r = 0.848$, $p < 0.01$). (Fig. 1C). FV1000 confocal laser microscopy showed strong association of the RFP expression within stroma (Fig. 2).

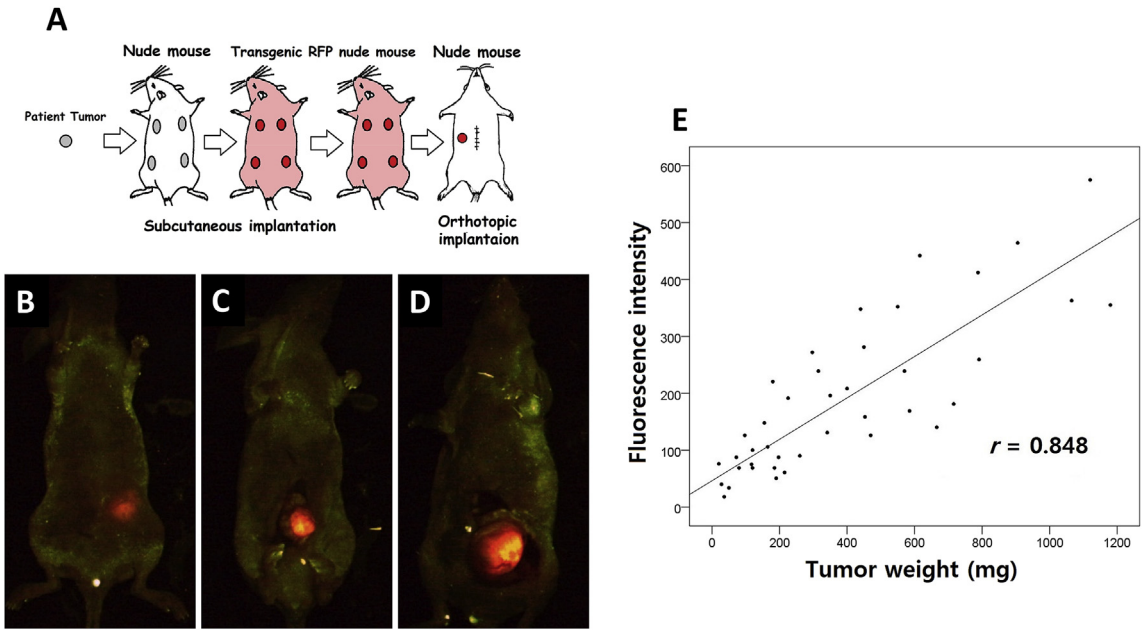

Fig. 1. (A) Experimental schema used to develop imageable PDOX models of human colon cancer. (B) Non-invasive red fluorescent protein (RFP) images of the PDOX primary colon cancer model (C) RFP image after laparotomy of a mouse after combination treatment (*o*-rMETase, 5-FU, OXA). (D) RFP image after laparotomy of a mouse in the untreated control group. (E) Correlation of fluorescence intensity with tumor weight. N = 10 mice/per treatment group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

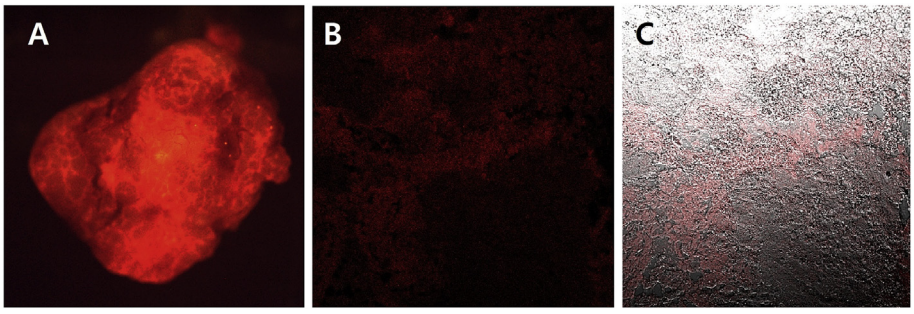

Fig. 2. FV1000® confocal laser microscope image of colon cancer in frozen section. (A) X 10, (B) x 60 and (C) Merged image, x 60.

3.2. Treatment efficacy of the combination of 5-FU + OXA + o-rMETase on the primary colon cancer PDOX model

At 6 weeks after implantation and completion of treatment, tumor weight was as follows: untreated control: 522.9 ± 323.2 mg; 5-FU + OXA: 255.6.9 ± 127.6 mg; *o*-rMETase: 296.1 ± 136.9 mg; combination of 5-FU + OXA with *o*-rMETase: 169.9 ± 80.3 mg 5-FU + OXA combined with *o*-rMETase was significantly more effective than *o*-rMETase alone and the untreated control ($p < 0.05$) (Fig. 3A). Relative tumor volume was as follows; untreated control: 3.54; 5-FU + OXA: 2.09; *o*-rMETase: 1.79; combination of 5-FU + OXA with *o*-rMETase: 1.33 (Fig. 3B). All treatments inhibited tumor growth compared to the untreated control group. 5-FU + OXA combined with *o*-rMETase was significantly more effective than *o*-rMETase alone or 5-FU + OXA alone ($p < 0.05$).

3.3. Tumor histology

Histologically, the untreated control tumor mainly comprised viable carcinoma cells (Fig. 3A, A′). In contrast, tumors treated with the combination of 5-FU, OXA and rMETase showed a great reduction of cancer cells as well as necrosis (Fig. 3B, B').

3.4. Intra-tumor MET levels

The intra-tumor MET levels of the untreated control group and the combination of 5-FU, OXA and rMETase group were compared. MET was significantly depleted by *o*-rMETase in combination with 5-FU and OXA ($p < 0.01$) (Fig. 4A). These results demonstrate that *o*-rMETase could deplete tumor MET levels despite the multiple sources of MET for the tumor including the diet, MET biosynthesis, and necrosis-related proteolysis.

3.5. Body weight

Body weight loss was observed in the 5-FU and OXA groups only. rMETase alone and the untreated control group did not have statistically significant body weight loss (Fig. 4B).

4. Discussion

Recently, a paper was published with the title “The new anti-cancer era: tumor metabolism targeting” [26]. However, this “new anticancer era” started in 1959 where Sugimura et al. [27] observed that depriving animals of MET arrested tumor growth. The Warburg

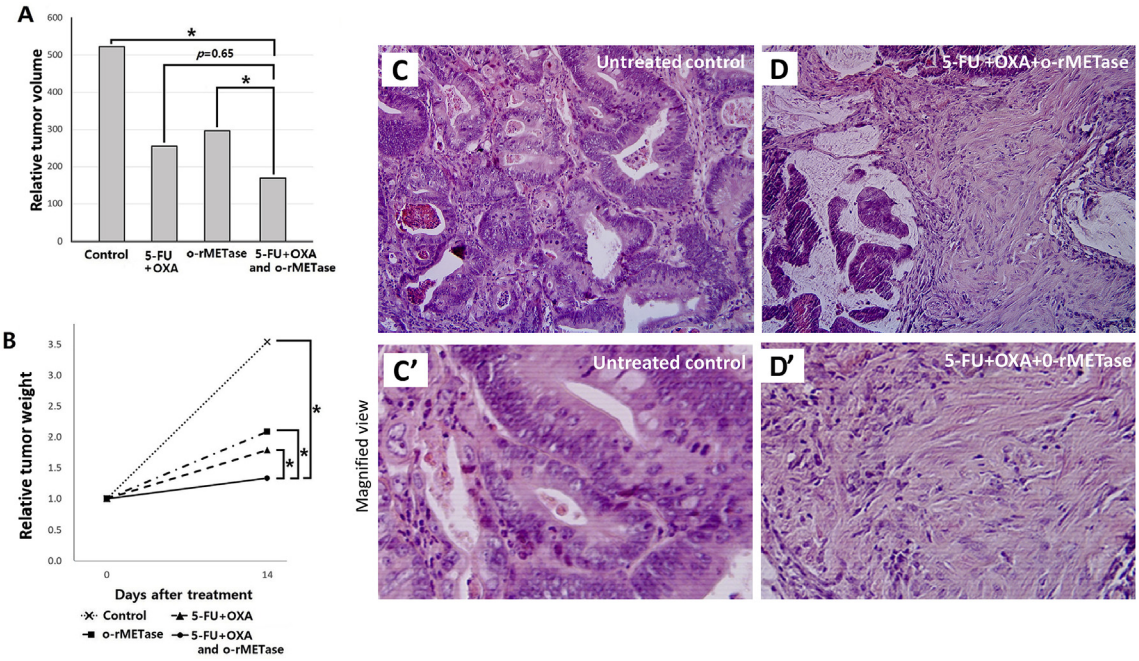

Fig. 3. Treatment efficacy of 5-FU + OXA, *o*-rMETase and their combination in the primary colon cancer PDOX model (A) relative tumor volume of treatment groups (B) tumor weight of treatment groups. $^{*}p < 0.05$. Tumor histology. (C, C′) untreated control (D, D′) combination treatment with 5-FU, OXA and *o*-rMETase. N = 10 mice/per treatment group.

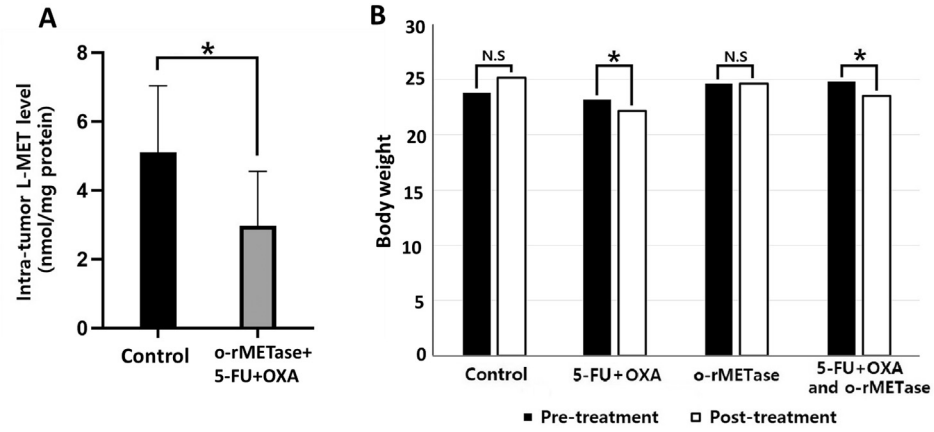

Fig. 4. (A) Intra-tumor MET levels. Bar graph show intra-tumor MET levels in control and 5-FU + OXA + *o*-rMETase combination groups. Error bars ± SD. $^{*}p < 0.05$. (B) Effect of 5-FU + OXA, rMETase and their combination on mouse body weight. $^{*}p < 0.05$. N = 10 mice/per treatment group.

effect refers to the significantly increased uptaked glucose by cancer cells. In addition to glucose restriction, specific amino acid restriction has been studied in the past to treat cancer. Cancer cells are more MET-dependent than normal cells, and simple dietary MR has been shown to reduce the proliferation of numerous cancer cell lines [28–31]. Recently, Wang et al. [1] demonstrated that high MET cycle activity causes high MET consumption leading to addiction to exogenous MET in cancer [1]. This is a phenomenon we discovered more than 40 years ago [2–4]. The inhibition of the MET cycle was enough to cripple the tumor-initiating capability [1]. Furthermore, MR is known to extend the life-span of various rat strains, indicating that basic health is not threatened by MR [32–34].

We have developed PDOX models of cancer for discovery of transformative therapy and for individualized therapy. o-rMETase could inhibit tumor growth in PDOX nude mouse models of various types of cancer [22–24]. Moreover, rMETase administered orally has little side effects. The present study suggests that *o*-rMETase used in combination with 5-FU and OXA was much more effective compared to any of these agents alone or rMETase has promise as a novel cancer therapeutic in combination with conventional chemotherapy for primary human colon cancer. Future studies will test this and other combinations with *o*-rMETase against additional important tumor types.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflicts of interest

JHP, TH, XC, NS, NS and RMH are or were unsalaried associates of AntiCancer Inc. MZ and QH are employees of AntiCancer Inc. AntiCancer Inc uses PDOX models for contract research and is

developing oral methioninase. The Authors declare that there are no other potential conflicts of interest.

Acknowledgements

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

References

[1] Z. Wang, L.Y. Yip, J.H.J. Lee, et al., Methionine is a metabolic dependency of tumor-initiating cells, Nat. Med. 25 (2019) 825–837.
[2] R.M. Hoffman, R.W. Erbe, High in vivo rates of methionine biosynthesis in transformed human and malignant rat cells auxotrophic for methionine, Proc. Natl. Acad. Sci. U. S. A 73 (1976) 1523–1527.
[3] D.W. Coalson, J.O. Mecham, P.H. Stern, et al., Reduced availability of endogenously synthesized methionine for S-adenosylmethionine formation in methionine-dependent cancer cells, Proc. Natl. Acad. Sci. U. S. A 79 (1982) 4248–4251.
[4] P.H. Stern, R.M. Hoffman, Elevated overall rates of transmethylation in cell lines from diverse human tumors, In Vitro 20 (1984) 663–670.
[5] P.H. Stern, J.O. Mecham, C.D. Wallace, et al., Reduced free-methionine in methionine-dependent SV40-transformed human fibroblasts synthesizing apparently normal amounts of methionine, J. Cell. Physiol. 117 (1983) 9–14.
[6] O. Warburg, The metabolism of carcinoma cells, J. Cancer Res. 9 (1925) 16.
[7] W. Xu, L. Gao, A. Shao, et al., The performance of 11C-Methionine PET in the differential diagnosis of glioma recurrence, Oncotarget 8 (2017) 91030–91039.
[8] R.M. Hoffman, Development of recombinant methioninase to target the general cancer-specific metabolic defect of methionine dependence: a 40-year odyssey, Expert Opin. Biol. Ther 15 (2015) 21–31.
[9] R.M. Hoffman, The wayward methyl group and the cascade to cancer, Cell Cycle 16 (2017) 825–829.
[10] L. Xin, J. Cao, H. Cheng, et al., Stealth cationic liposomes modified with anti-CAGE single-chain fragment variable deliver recombinant methioninase for gastric carcinoma therapy, J. Nanosci. Nanotechnol. 13 (2013) 178–183.
[11] L. Xin, H.T. Zhang, W.F. Yang, et al., Evaluation of METase-pemetrexed-loaded PEG-PLGA nanoparticles modified with anti-CD133-scFV for treatment of gastric carcinoma, Biosci. Rep. 38 (2018).
[12] Y. Tan, M. Xu, R.M. Hoffman, Broad selective efficacy of recombinant methioninase and polyethylene glycol-modified recombinant methioninase on cancer cells in Vitro, Anticancer Res. 30 (2010) 1041–1046.
[13] W. Kreis, C. Hession, Biological effects of enzymatic deprivation of L-methionine in cell culture and an experimental tumor, Cancer Res. 33 (1973) 1866–1869.
[14] S. Yano, K. Takehara, M. Zhao, et al., Tumor-specific cell-cycle decoy by Salmonella typhimurium A1-R combined with tumor-selective cell-cycle trap by methioninase overcome tumor intrinsic chemoresistance as visualized by FUCCI imaging, Cell Cycle 15 (2016) 1715–1723.
[15] S. Yano, S. Li, Q. Han, et al., Selective methioninase-induced trap of cancer cells in S/G2 phase visualized by FUCCI imaging confers chemosensitivity, Oncotarget 5 (2014) 8729–8736.
[16] S. Yano, Y. Zhang, M. Zhao, et al., Tumor-targeting Salmonella typhimurium A1-R decoys quiescent cancer cells to cycle as visualized by FUCCI imaging and become sensitive to chemotherapy, Cell Cycle 13 (2014) 3958–3963.
[17] K. Kawaguchi, K. Igarashi, S. Li, et al., Combination treatment with recombinant methioninase enables temozolomide to arrest a BRAF V600E melanoma in a patient-derived orthotopic xenograft (PDOX) mouse model, Oncotarget 8 (2017) 85516–85525.
[18] K. Kawaguchi, K. Igarashi, S. Li, et al., Recombinant methioninase (rMETase) is an effective therapeutic for BRAF-V600E-negative as well as -positive melanoma in patient-derived orthotopic xenograft (PDOX) mouse models, Oncotarget 9 (2018) 915–923.
[19] K. Igarashi, S. Li, Q. Han, et al., Growth of doxorubicin-resistant undifferentiated spindle-cell sarcoma PDOX is arrested by metabolic targeting with recombinant methioninase, J. Cell. Biochem. 119 (2018) 3537–3544.
[20] K. Igarashi, K. Kawaguchi, T. Kiyuna, et al., Tumor-targeting Salmonella typhimurium A1-R combined with recombinant methioninase and cisplatinum eradicates an osteosarcoma cisplatinum-resistant lung metastasis in a patient-derived orthotopic xenograft (PDOX) mouse model: decoy, trap and kill chemotherapy moves toward the clinic, Cell Cycle 17 (2018) 801–809.
[21] K. Kawaguchi, K. Miyake, Q. Han, et al., Targeting altered cancer methionine metabolism with recombinant methioninase (rMETase) overcomes partial gemcitabine-resistance and regresses a patient-derived orthotopic xenograft (PDOX) nude mouse model of pancreatic cancer, Cell Cycle 17 (2018) 868–873.
[22] K. Kawaguchi, K. Miyake, Q. Han, et al., Oral recombinant methioninase (o-rMETase) is superior to injectable rMETase and overcomes acquired gemcitabine resistance in pancreatic cancer, Cancer Lett. 432 (2018) 251–259.
[23] T. Higuchi, K. Kawaguchi, K. Miyake, et al., Oral recombinant methioninase combined with caffeine and doxorubicin induced regression of a doxorubicin-resistant synovial sarcoma in a PDOX mouse model, Anticancer Res. 38 (2018) 5639–5644.
[24] K. Kawaguchi, T. Higuchi, S. Li, et al., Combination therapy of tumor-targeting Salmonella typhimurium A1-R and oral recombinant methioninase regresses a BRAF-V600E-negative melanoma, Biochem. Biophys. Res. Commun. 503 (2018) 3086–3092.
[25] A. Suetsugu, M. Katz, J. Fleming, et al., Non-invasive fluorescent-protein imaging of orthotopic pancreatic-cancer-patient tumorgraft progression in nude mice, Anticancer Res. 32 (2012) 3063–3067.
[26] A. Borriello, F. Della Ragione, The new anticancer era: tumor metabolism targeting, Cell Cycle 16 (2017) 310–311.
[27] T. Sugimura, S.M. Birnbaum, M. Winitz, et al., Quantitative nutritional studies with water-soluble, chemically defined diets. VIII. The forced feeding of diets each lacking in one essential amino acid, Arch. Biochem. Biophys. 81 (1959) 448–455.
[28] H. Jeon, J.H. Kim, E. Lee, et al., Methionine deprivation suppresses triple-negative breast cancer metastasis in vitro and in vivo, Oncotarget 7 (2016) 67223–67234.
[29] F. Poirson-Bichat, G. Gonfalone, R.A. Bras-Goncalves, et al., Growth of methionine-dependent human prostate cancer (PC-3) is inhibited by ethionine combined with methionine starvation, Br. J. Canc. 75 (1997) 1605–1612.
[30] D. Komninou, Y. Leutzinger, B.S. Reddy, et al., Methionine restriction inhibits colon carcinogenesis, Nutr. Cancer 54 (2006) 202–208.
[31] E. Strekalova, D. Malin, D.M. Good, et al., Methionine deprivation induces a targetable vulnerability in triple-negative breast cancer cells by enhancing TRAIL receptor-2 expression, Clin. Cancer Res. 21 (2015) 2780–2791.
[32] J.A. Zimmerman, V. Malloy, R. Krajcik, et al., Nutritional control of aging, Exp. Gerontol. 38 (2003) 47–52.
[33] N. Orentreich, J.R. Matias, A. DeFelice, et al., Low methionine ingestion by rats extends life span, J. Nutr. 123 (1993) 269–274.
[34] R.M. Hoffman, Methionine restriction and life-span extension, Methods Mol. Biol. 1866 (2019) 263–266.
[35] K. Miyake, T. Kiyuna, M. Miyake, et al., Gemcitabine combined with docetaxel precisely regressed a recurrent leiomyosarcoma peritoneal metastasis in a patient-derived orthotopic xenograft (PDOX) model, Biochem. Biophys. Res. Commun. 509 (2019) 1041–1046.
[36] X.Y. Fu, J.M. Besterman, A. Monosov, et al., Models of human metastatic colon cancer in nude mice orthotopically constructed by using histologically intact patient specimens, Proc. Natl. Acad. Sci. U. S. A 88 (1991) 9345–9349.
[37] Y. Tan, M. Xu, X. Tan, et al., Overexpression and large-scale production of recombinant L-methionine-alpha-deamino-gamma-mercaptomethane-lyase for novel anticancer therapy, Protein Expr. Purif. 9 (1997) 233–245.
[38] T. Murakami, S. Li, Q. Han, et al., Recombinant methioninase effectively targets a Ewing's sarcoma in a patient-derived orthotopic xenograft (PDOX) nude-mouse model, Oncotarget 8 (2017) 35630–35638.
[39] X. Sun, Y. Tan, Z. Yang, et al., A rapid HPLC method for the measurement of ultra-low plasma methionine concentrations applicable to methionine depletion therapy, Anticancer Res. 25 (2005) 59–62.