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Survival efficacy of the combination of the methioninase gene and methioninase in a lung cancer orthotopic model

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We have previously demonstrated the antitumor efficacy of recombinant methioninase (rMETase) derived from *Pseudomonas putida*. To enhance the efficacy of rMETase, we have constructed the pLGFP-METSN retrovirus encoding the *P. putida* methioninase (MET) gene fused with the green fluorescent protein (GFP) gene. pLGFP-METSN or control vector pLGFPSN was introduced into the human lung cancer cell line H460. The methionine level of H460-GFP-MET cells was reduced to 33% of that of H460-GFP cells. rMETase (0.08 U/mL) in the medium resulted in 10% survival of H460-GFP-MET cells compared with untreated cells *in vitro*. In contrast, rMETase-treated H460-GFP cells survived at 90% of control. Tissue fragments harvested from subcutaneous tumors of H460-GFP-MET or H460-MET were implanted by surgical orthotopic implantation into the lungs of nude mice. A suboptimal dose of rMETase was administered intraperitoneally daily to mice in each group. Overall survival of rMETase-treated animals with H460-GFP-MET tumors was significantly longer than either rMETase-treated or -untreated animals with H460-GFP tumors ($P < .05$ in log-rank test). In two repeat experiments, rMETase-treated animals with H460-GFP-MET tumors had a 30-day survival of 80% and 83%, respectively. Untreated animals with H460-GFP-MET tumors had a 30-day survival of 40% and 58%, respectively. rMETase-treated animals with H460-GFP tumors had a 30-day survival of 0% and 33%, respectively. Untreated animals with H460-GFP tumors had a 30-day survival of 0% and 33%, respectively. The retrovirus-mediated gene transfer of METase decreased the intracellular methionine level of tumor cells and consequently enhanced the efficacy of treatment with the rMETase protein. We have thus demonstrated a new strategy of combination tumor therapy with the gene and gene product of MET. **Cancer Gene Therapy (2000) 7, 332–338**

Key words: Methionine dependence; recombinant methioninase; methioninase gene; retrovirus; green fluorescent protein; fusion gene.

A number of different strategies for cancer gene therapy have been developed. For example, the p53 gene has been shown to inhibit tumor cell growth and induce apoptosis.[1] The antitumor efficacy of the p53 gene using retroviral,[2] adenoviral,[3] or lipid-based delivery[4] has been demonstrated. However, this strategy is limited to tumors with defective p53. The herpes simplex virus thymidine kinase gene (HSV-TK) in combination with ganciclovir has also been shown to inhibit tumor growth.[5] This prodrug approach activates ganciclovir by TK-mediated phosphorylation. The retroviral vector encoding HSV-TK is selectively expressed in dividing cells such as those in the tumor. Tumor regression has been shown even when only a small number of cells have been transfected by retroviral HSV-TK in what is called a bystander effect.[6–9] Viral transfer of the cytosine deaminase gene, which confers fluorocytosine sensitivity, is also being used in gene therapy experiments.[6,10,11] Other gene therapy strategies for cancer include the retinoblastoma gene,[12] the E1a gene,[13] and the ras gene.[14] Tissue-specific promoters are being used to target the expression of therapeutic genes to tumor cells.[15–17] Other approaches to gene therapy have included manipulating the immune system with genes such as interleukin-2.[6] Autologous tumor cells transduced with a cytokine gene are being used as vaccines.[6,18–22]

This report describes a new approach to gene therapy that targets the elevated methionine dependence of tumor cells[23] with the methioninase (MET) gene derived from *Pseudomonas putida*. The elevated methionine requirement for cell growth has been shown to be present in the majority of cancer cells compared with normal cells.[23–32] There have been several therapeutic strategies developed to target the methionine depen-

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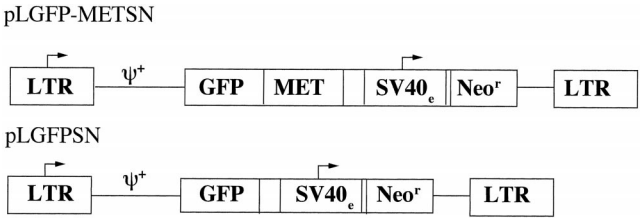

Figure 1. Schematic illustrations of the plasmids used in this study.

dence of tumor cells. Methionine starvation therapy using a methionine-free diet or total parenteral nutrition prolonged the survival time of tumor-bearing rodents[33–35] and high-stage gastric cancer patients.[36] The recombinant MET (rMETase) protein, cloned from *P. putida* and produced in *Escherichia coli*, has been demonstrated to have antitumor efficacy *in vitro* and *in vivo*.[32,37–39]

We postulated that transfer of the MET gene into tumor cells should decrease the intracellular methionine level and result in an increase in methionine dependence.

MATERIALS AND METHODS

Cell lines and animals

The human non-small cell lung adenocarcinoma cell line H460 was maintained in RPMI 1640 with 10% fetal bovine sera (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. The NIH-3T3-based amphotropic retroviral packaging cell line PT67 was purchased from Clontech Laboratories (Palo Alto, Calif) and maintained in Dulbecco's modified Eagle's medium with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Male and female athymic BALB/c nude mice between 4 and 6 weeks of age were used in this study. The animals were bred and maintained in a high-efficiency particulate air-filtered environment. Cages, food, and bedding were sterilized by autoclaving. The breeding pairs were obtained from Charles River Laboratories (Wilmington, Mass). The animal diets were obtained from Harlan Teklad (Madison, Wis). A total of 5.0% (vol/vol) ampicillin (Sigma, St. Louis, Mo) was added to the autoclaved drinking water. 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 Laboratory Animals under assurance number A3873-1.

Plasmid construction

We constructed pLGFP-METSN and pLGFPSN, which were both based on the pLXSN plasmid. Figure 1 represents the structure of both vectors. pLGFP-METSN was generated by inserting the GFP-MET gene, which is a fusion of the jellyfish *Aequorea victoria* green fluorescent protein (GFP)[40] with the MET gene derived from *P. putida*.[37] MET was amplified by polymerase chain reaction using the pAC-1 rMETase gene clone[37] as a template and two oligonucleotide primers: 5′-CCGCTCGAGATGCACGGCTCCAACAAGCTCCCA-3′ and 5′-CGCGGATCCTTAGGCACTCGCCTTGAGTGCCTG-3′.

The 1.2-kb MET DNA was ligated into the pGFPC-3 (Clontech) plasmid vector downstream of the GFP gene at the *Xho*I/*Bam*HI site. The resulting plasmid was digested by *Nhe*I, blunt-ended, and digested with *Bam*HI. The retroviral expression vector pLXSN (Clontech) was also digested by *Hpa*I, blunt-ended, and digested by *Bam*HI. The GFP-MET cassette was ligated into pLXSN at the blunted *Hpa*I and *Bam*HI site. The GFP-MET gene was driven by the Moloney murine leukemia virus long terminal repeat. The neomycin resistance gene was driven by the simian virus 40 early promoter gene.

pLGFPSN was constructed by inserting the GFP gene purified from pGFP-1 (Clontech) into the pLXSN retroviral vector.

Retrovirus preparation

PT67 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin. For retroviral vector production, packaging cells at 70% confluence in six-well culture dishes were transfected with DOTAP™ (Boehringer Mannheim, Mannheim, Germany) liposomal reagent containing saturating amounts of pLGFPSN or pLGFP-METSN plasmid. After 18 hours of incubation, the culture medium was replaced with fresh medium. For the selection of stable virus-producing cells, the cells were cultured in the presence of 0.8 mg/mL G418 (Life Technologies, Grand Island, NY) at 2 days posttransfection for an additional 2 weeks.

Retroviral transduction of H460 cells

H460 cells at 20% confluence were washed and exposed to PT67/pLGFP-METSN or pLGFPSN supernatants at a 1:1 ratio of RPMI 1640 medium containing 10% FBS in the presence of 8 μg polybrene for 12 hours. The cells were then maintained in fresh RPMI 1640 containing 400 μg/mL G418 to select for transduced cells. The expression level of both vectors was monitored by GFP fluorescence. The high-expression clones with each vector were isolated using cloning cylinders and amplified for further experiments.

METase activity assay

The METase activities of the H460-transduced cells were measured according to the method of Tanaka et al.[41] Briefly, 10^7cells were collected after trypsin-ethylenediaminetetraacetic acid digestion. Cell pellets were washed with phosphate-buffered saline and diluted with 0.5 mL of phosphate-buffered saline. The cells were homogenized by sonication for 1 minute with centrifugation at 14,000 rpm for 10 minutes. METase activity was measured in the supernatant by determining α-ketobutyrate production from 10 mM methionine using 3-methyl-2-benzo-thiazoline hydrazone. The amount of reaction product was measured with a Hitachi model U-2000 spectrophotometer (Hitachi, Tokyo, Japan) at 335 nm. The amount of protein in the cell lysate was determined with the Lowry Reagent Kit (Sigma) using bovine serum albumin as a standard. Specific METase activity was calculated as units per milligram of protein, with 1 U of enzyme defined as the amount that catalyzed the formation of 1 μmol of α-ketobutyrate per minute.

Methionine levels of tumor cells

The methionine level in the cell lysates was determined by high performance liquid chromatography (Hitachi L 6200A Intelligent pump) after derivatization of amino acids with the fluorescent reagent *o*-phthaldialdehyde (OPA).[42,43] The cell lysate (200 μL) was first precipitated by acetonitrile (800 μL). A total of 10 μL of supernatant was then mixed with 5 μL of OPA. After 1 minute, 50 μL of 0.1 M sodium acetate (pH 7.0)

was added, and 20 μL of sample were loaded on a reversed-phase Supercosil LC-18-DB (Supelco, Bellefonte, PA) column at room temperature. The amino acid derivatives were resolved with solution A (tetrahydrofuran/methanol/0.1 M sodium acetate (pH 7.2): 5/95/900) and solution B (methanol). A gradient from 20% to 60% was run at a flow rate of 1.5 mL/minute. The eluate was read with a fluorescence spectrophotometer (Hitachi F1000) at 350–450 nm.

In vitro *efficacy of rMETase*

The 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT) assay[44] was used to determine the *in vitro* growth inhibition of tumor cells by rMETase treatment. Cells (2000 cells/well) were plated in 96-well culture plates in 100 μL of culture medium. After 24 hours, various concentrations of rMETase (100 μL) were added to the wells. After 3 days of incubation with rMETase, cytotoxicity was measured with the MTT assay. MTT was used at a concentration of 0.2 μg/μL with incubation for 2 hours. The resulting formazan product was solubilized in dimethylsulfoxide and absorbance was measured at 540 nm in a BioRad Benchmark Microplate Reader (BioRad Laboratories, Hercules, Calif) using 1-cm pathlength plastic spectrophotometer cells. Growth inhibition was expressed as the percentage of absorbance of treated cultures with respect to untreated cells.

In vivo *evaluation of the efficacy of rMETase*

Fragments (1 mm in diameter) derived from H460-GFP or H460-GFP-MET tumors growing subcutaneously in nude mice were implanted by surgical orthotopic implantation (SOI) into the left lungs of nude mice.[45,46]

From days 2–13 postoperation, 100 U of rMETase were administered intraperitoneally twice a day. At day 17 postoperation, four to five mice in each group were chosen randomly and sacrificed. The primary tumor weights were measured and the incidence of metastasis in relevant organs was evaluated under fluorescence microscopy of fresh tissue. Statistical significance for tumor weights was determined with the Student's *t* test. The remaining 10–12 mice in each group were monitored for survival. This experiment was repeated one time. Differences in survival were compared at 30 days. Overall survival was analyzed with the log-rank test.

RESULTS AND DISCUSSION

Transduction of H460 with GFP-MET or GFP genes

H460 cells transduced with either the GFP-MET or GFP genes were able to grow *in vitro*. High-expression clones containing these vectors were isolated and selected by the extent of their GFP fluorescence. The GFP fluorescence was evenly distributed in cells with the GFP gene. In contrast, in GFP-MET cells, the fluorescence was localized only in the cytoplasm and not in the nuclei. The reason for localization of GFP-MET expression only in the cytoplasm remains unclear. There were no significant differences in the cell growth rates between GFP cells and GFP-MET cells as determined by comparing their doubling time *in vitro* (data not shown).

METase activity and methionine levels

The METase activity of GFP-MET cells was 33 mU/mg protein (Table 1). METase activity was not detectable in the H460 parent cells or in H460-GFP cells. Pure rMETase has a specific activity of ~20 U/mg protein, which means that the GFP-MET cells expressed METase at ~0.17% of their total protein. The free methionine level of H460-GFP-MET was determined to be ~33% of that of H460-GFP cells or H460 parent cells (Table 1).

Table 1. MET Activity and Free Methionine Levels of MET-Transduced H460 Lung Tumor Cells *In Vitro

	MET activity	Free methionine level
H460	0.75 mU/mg protein	30.2 nmol/mg protein
H460-GFP	1.5 mU/mg protein	33.0 nmol/mg protein
H460-GFP-MET	33 mU/mg protein	10.1 nmol/mg protein

*Methioninase activity was measured with 3-methyl-2-benzothiazoline hydrazone. Cellular free methionine levels were measured by OPA-derivitized amino acids separated by high performance liquid chromatography. Please see *Materials and Methods* for a description of procedures.

In vitro *efficacy of rMETase*

The efficacy of rMETase *in vitro* was determined with the MTT assay. H460-GFP-MET cells were more sensitive to low concentrations of rMETase compared with H460-GFP cells or H460 cells (Fig 2). In the presence of 0.08 U/mL rMETase, the survival of H460-GFP-MET cells was 10% compared with the untreated cells. In contrast, H460 and H460-GFP cells were only slightly affected by this concentration of rMETase (Fig 2). These results indicated that the MET gene increased the methionine requirement of these tumor cells, making them more sensitive to rMETase.

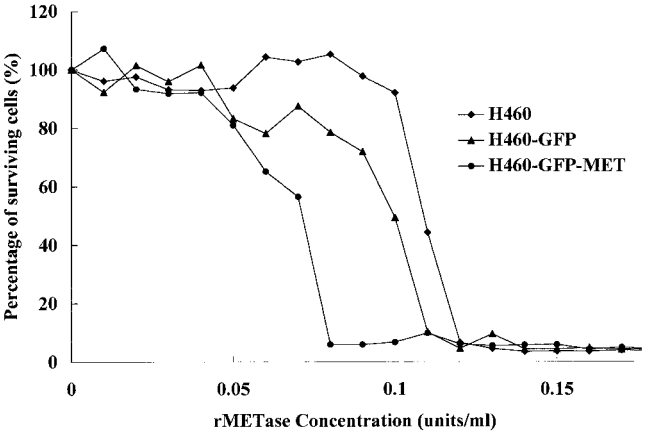

Figure 2. *In vitro* efficacy of rMETase on H460-GFP-MET cells, H460-GFP cells, and H460 parent cells. Cytotoxicity was assayed with the MTT assay. The IC_{50} was 0.07 U/mL for GFP-MET cells, 0.115 U/mL for H460 cells, and 0.098 U/mL for GFP cells. In the presence of 0.08 U/mL of rMETase, GFP-MET cell survival was only 10% compared with untreated cells. In contrast, the survival of H460 parental cells and H460-GFP cells were only slightly sensitive to this concentration of rMETase.

Table 2. *In Vivo* Efficacy of the MET Gene and rMETase Protein on Primary Tumor Weights and Metastatic Potential of H460 Lung Tumors*

Group	Primary tumor weights (grams)†	Contralateral lung	Contralateral pleural dissemination	Mediastinal lymph node	Direct invasion to vertebrae	Number of mice with no metastasis
GFP tumor, treated	0.927 ± 0.135	4/4	2/4	4/4	3/4	0/4
GFP tumor, rMETase-treated	0.509 ± 0.260	4/4	0/4	4/4	2/4	0/4
GFP-MET tumor, untreated	0.692 ± 0.307	4/5	3/5	4/5	3/5	1/5
GFP-MET tumor, rMETase-treated	0.411 ± 0.169	3/5	2/5	3/5	2/5	2/5

*Fragments (1 mm in diameter) derived from H460-GFP or H460-GFP-MET tumors growing subcutaneously in nude mice were implanted by SOI into the left lung of nude mice.[45,46] From day 2 to day 13 postoperation, 100 U of rMETase were administered intraperitoneally twice a day. At day 17 postoperation, four to five mice in each group were chosen randomly and sacrificed. The primary tumor weights were measured and the incidence of metastasis in relevant organs was evaluated by fluorescence microscopy of fresh tissue. Statistical significance was determined with the Student's *t* test.

†Significant difference between untreated GFP tumors and rMETase-treated GFP tumors ($P < .05$) and between untreated GFP tumors and rMETase-treated GFP-MET tumors ($P < .005$).

In vivo *efficacy of rMETase*

We used the SOI model to test the efficacy of rMETase *in vivo*, because the SOI model allows extensive metastasis and a resulting short survival time.[45,46] The primary lung tumor weights were measured at 17 days after SOI. The mean ± SD of tumor weights was 0.927 ± 0.135 g for untreated GFP tumors, 0.509 ± 0.260 g for rMETase-treated GFP tumors, 0.692 ± 0.307 g for untreated GFP-MET tumors, and 0.411 ± 0.169 g for rMETase-treated GFP-MET tumors (Table 2). There were significant differences in weight between the untreated GFP tumors and rMETase-treated GFP tumors ($P = .05$). rMETase-treated GFP-MET tumors were the smallest, but the difference from untreated GFP-MET tumors was not significant ($P = .11$). The MET gene alone in the H460-GFP-MET tumors may itself have partially showed tumor growth.

We examined the metastatic potential of both tumors under fluorescence microscopy. Metastases to mediastinal lymph nodes (Fig 3, A and B) and contralateral lungs (Fig 3C) were observed. The frequencies of these metastases were somewhat decreased in the rMETase-treated GFP-MET group, which was the only group that had two animals with no metastasis. The tumor growth and metastasis results are summarized in Table 2.

Animals with GFP-MET tumors treated with rMETase had the longest median survival time among the four groups in two repeat experiments. Untreated animals with an H460-GFP tumor had a median survival of 23 and 25 days, respectively. rMETase-treated animals with an H460-GFP tumor had a median survival of 22 and 24 days, respectively. Untreated animals with an H460-GFP-METase tumor had a median survival of 27 and 32 days, respectively. rMETase-treated animals with an H460-GFP-MET tumor had a median survival of 33 and 36 days, respectively. Both experiments demonstrated a 50% increase in the survival of the rMETase-treated animals with H460-GFP-MET tumors over rMETase-treated animals with H460-GFP tumors. The overall survival of rMETase-treated animals with H460-GFP-MET tumors was significantly longer than either rMETase-treated or -untreated animals with H460-GFP tumors ($P < .05$ in log-rank test) (Fig 4).

In two repeat experiments, rMETase-treated animals with H460-GFP-MET tumors had a 30-day survival of 80% and 83%, respectively. Untreated animals with H460-GFP-MET tumors had a 30-day survival of 40% and 58%, respectively. rMETase-treated animals with H460-GFP tumors had a 30-day survival of 0% and 33%, respectively. Untreated animals with H460-GFP tumors had a 30-day survival of 0% and 33%, respectively. The difference in 30-day survival between the rMETase-treated GFP and rMETase-treated GFP-MET groups had *P* values of $<.005$ in both experiments. The difference in 30-day survival between the rMETase-treated GFP-MET group and the untreated GFP-MET group had a *P* value of $<.05$ in experiment 1 and a *P* value of <0.08 in experiment 2 (Table 3). The data demonstrate that the addition of rMETase increased the survival of the GFP-MET group. This finding is consistent with the increased sensitivity of the H460-GFP-MET cells to rMETase compared with controls without the MET gene (Fig 2).

The GFP-MET fusion gene has the following advantages: (a) The expression levels of MET can be monitored by fluorescence in living cells and tissues and (b) the transduced tumor cell behavior *in vivo* can be easily visualized by GFP fluorescence such as metastasis formation (Fig 3).[47–49]

A retroviral vector has also been constructed by Aran et al[50] with GFP and with the multidrug resistance gene that has been used *in vitro* as a fluorescent vector and is proposed for future *in vivo* studies.

MET gene transfer in combination with rMETase prolonged the survival time of tumor-bearing mice. Because we selected surviving cells after transduction of the GFP-MET gene, the extent of the MET expression in these cells may be limited. Thus the efficacy of the MET gene was enhanced by exogenous rMETase.

In this study, a single selected cell line, H460, was characterized both *in vitro* and *in vivo*. However, a series of seven H460-GFP-MET clones had significantly lower

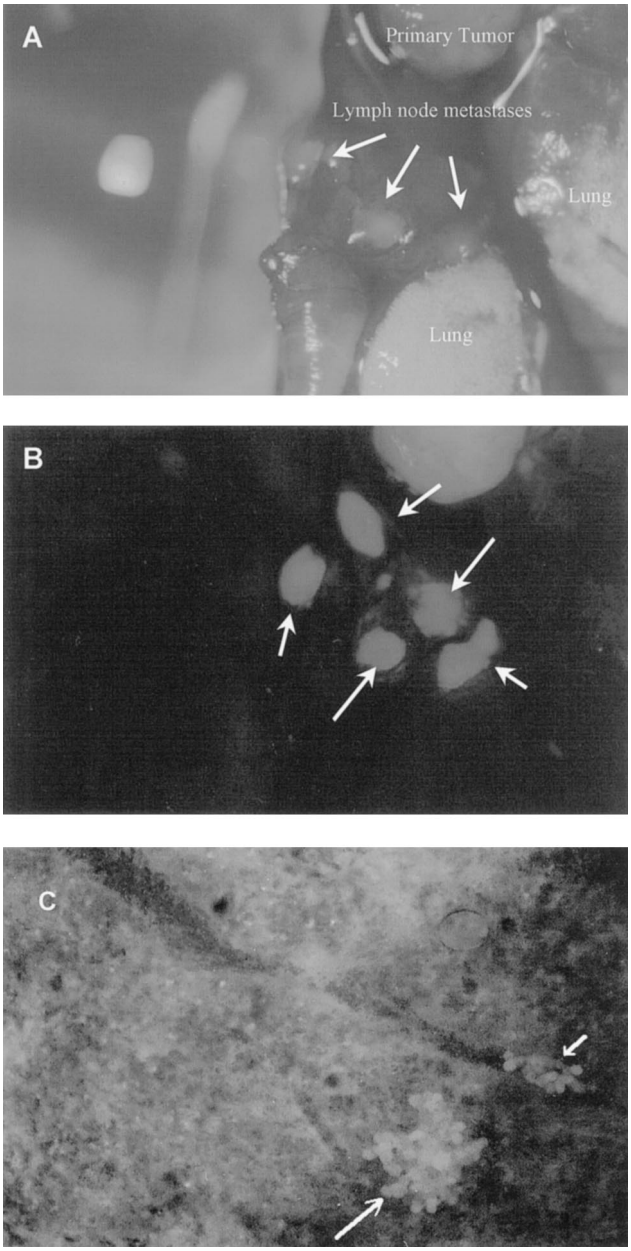

Figure 3. Orthotopic growth and metastases as visualized by GFP: Lymph node metastases of the left lung hilus of an H460-GFP-MET tumor. **A:** Bright field microscopy. Arrows indicate lymph node metastases. **B:** Same field viewed under fluorescence with GFP cube. Primary tumor and lymph nodes are brightly visualized. Arrows indicate lymph node metastases. **C:** Early stage contralateral lung metastasis of an H460-GFP tumor. Tumor embolus in a small vessel (small arrow) and a small tumor colony (large arrow) are visualized under fluorescence with GFP cube.

rMETase 50% inhibitory concentrations (IC_{50}s) than a series of four H460-GFP clones ($P < .005$) (data not shown). Whether the strategy of combination therapy with the MET gene and rMETase protein would work with other tumor cell lines is open to question at this time. The toxicity of the MET gene in normal cells has not yet been demonstrated. However, we have described

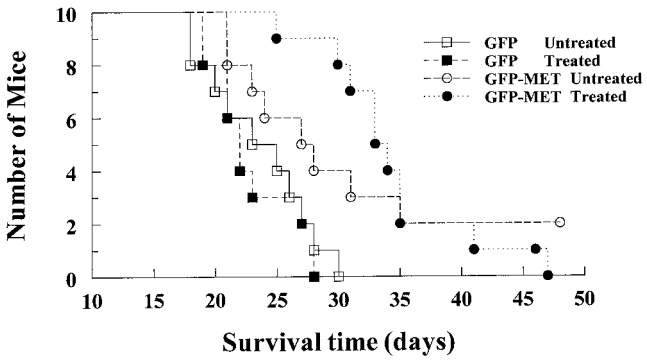

Figure 4. Survival curves of four groups of mice orthotopically implanted with H460. Untreated animals with an H460-GFP tumor had a median survival of 23 days. rMETase-treated animals with an H460-GFP tumor had a median survival of 22 days. Untreated animals with an H460-GFP-MET tumor had a median survival of 27 days. rMETase-treated animals with an H460-GFP-MET tumor had a median survival of 33 days, a 50% increase over rMETase-treated animals with H-460-GFP tumors. Overall survival of rMETase-treated animals with H460-GFP-MET tumors was significantly longer than either rMETase-treated or -untreated animals with H460-GFP tumors ($P < .05$ in log-rank test).

the efficacy and toxicity of rMETase in a series of human tumor cell lines and normal cell strains.[32] This study included seven different tumor types for a total of >20 tumor cell lines. The tumor selectivity of rMETase was shown to be very large in this study, with the IC_{50}s of the tumor cells lower than that of the normal cells by as much as one order of magnitude. It is thus surmised that the MET gene could have a similar broad range of tumor efficacy and degree of tumor selectivity. This remains to be tested definitively in future experiments *in vivo*.

We have thus demonstrated a new strategy of combination tumor therapy with the MET gene and its gene product. Future experiments will involve direct *in vivo* administration of the MET gene both with and without rMETase to tumor-bearing animals.

Table 3. *In Vivo* Efficacy of the MET Gene and rMETase Protein on the 30-Day Survival Rate of Mice Implanted with H460 Lung Tumors

Animal group	Percentage of surviving animals	
	Experiment 1	Experiment 2
GFP tumor, untreated	0%	33.33%
GFP tumor, rMETase-treated	0%**	33.33%**
GFP-MET tumor, untreated	40%*	58.33%†
GFP-MET tumor, rMETase-treated	80%*	83.33%†

100 U of rMETase were administered intraperitoneally twice a day as described for Table 2. A total of 10–12 mice in each group were monitored for survival. Differences in survival at 30 days were analyzed with the χ^2 test.

*, $P < .05$; **, $P < .005$; †, $P = .08$; all compared with GFP-MET tumor, rMETase-treated.

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