MET Expressed in Glioma Stem Cells Is a Potent Therapeutic Target for Glioblastoma Multiforme

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Abstract. *Background: Glioblastoma multiforme (GBM) is the most frequent and the most malignant tumor among adult brain tumors. Previous reports led us to hypothesize that the proto-oncogene mesenchymal–epithelial transition (MET) expressed in glioma stem cell-like cells (GSCs) would be a potent therapeutic target for GBM. Patients and Methods: To address this question, we analyzed 113 original samples of tumors from patients based on immunohistochemistry. During this process, we were able to establish GSC lines from patients with GBM that were MET-positive and MET-negative. Using these cells, we tested the therapeutic impact of a MET inhibitor, crizotinib, both in vitro and in vivo. Results: Patients with MET-positive GBM exhibited poor survival. GSC-based experiments revealed that treatment with crizotinib, both in vitro and in vivo, exhibited therapeutic efficacy particularly against MET-positive GSCs. Conclusion: Based on these findings, we conclude that MET expressed in GSCs might be a potent therapeutic target for GBM.*

Gliomas are the most frequent type of primary brain tumor in adults. Among them, glioblastoma multiforme (GBM) is the most malignant. The standard therapy for GBM includes surgical removal, radiotherapy, and chemotherapy based on temozolomide. GBMs heavily infiltrate into the neighboring brain parenchyma, and they are highly refractory to chemotherapeutic agents (1). As a result, the median survival of GBM patients is no longer than 14.6 months despite maximal therapy (2). Development of novel therapeutic strategies for GBM is therefore urgent.

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Mesenchymal-epithelial transition (MET), also known as hepatocyte growth factor receptor (HGFR), functions as a receptor for hepatocyte growth factor (HGF). It is a membrane receptor tyrosine kinase and expressed in epithelial cells, liver, pancreas, prostate, kidney, and bone marrow (3). The MET–HGF signal induces cell division, growth, and cell migration (3). *MET* is also a known proto-oncogene for a variety of malignant tumors, including small cell lung cancer, breast carcinoma, prostatic carcinoma, hepatocellular carcinoma, as well as GBM (4). MET has also been associated with the stem cell phenotype in glioma by regulating sphere formation, cell proliferation, and differentiation (4). Particularly in GBM, MET has been suggested to be important for tumor cell survival, angiogenesis and invasion (5). Based on these findings, MET is believed to be a therapeutic candidate for malignant gliomas.

In recent years, numerous studies have reported the presence of stem cell-like cells in solid tumors (6). Likewise, GBM is known to possess glioma stem cell-like cells (GSCs) (7). Based on a line of previous studies (8), GSCs are defined as pluripotent cells that express the stem cell marker cluster of differentiation 133 (CD133) and nestin, an intermediate filament protein found at high levels in undifferentiated central nervous system cells. In addition, they are known to exhibit resistance to chemotherapy and play a critical role in repopulating tumor masses. That is, although chemotherapy can kill the majority of glioma cells and induce a temporary regression, it ultimately results in disease relapse by the enrichment of chemoresistant GSCs (9). To achieve long-lasting remission of GBM, we therefore, need to develop new therapeutic approaches that target both the tumor bulk and the GSC compartment.

Based on these findings, we hypothesized that MET expressed in GSCs would be a potent therapeutic target for GBM. To address this question, we analyzed our patient cohort using immunohistochemistry (IHC). We also

established GSC lines that were MET-positive and MET-negative from patients with GBM and tested the efficacy of targeting MET with the inhibitor crizotinib, both *in vitro* and *in vivo*.

Materials and Methods

Patients and mice. Patient-relevant experiments were approved by the Institutional Review Board of Kindai University (10). This study included 113 patients with the World Health Organization (WHO) grade II-IV glioma who were referred to Kindai University Hospital between January 2008 and December 2014. All the patients underwent initial surgery during this period; no treatment was performed prior to surgical resection. Clinical and pathological data including gender, age, mindbomb E3 ubiquitin protein ligase 1 (MIB1), overall survival (OS), *MET* status, and tumor differentiation were collected (Table I). All tumor samples were classified by two neuropathologists according to the WHO classification (11).

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of Kindai University (12). As there are gender-related differences in the pharmacokinetics and toxicity of crizotinib in mice (13), only female mice were used in these experiments. Six- to eight-week-old NOD-SCID mice were obtained from Charles River (Tokyo, Japan) and maintained under specific pathogen-free conditions at the Animal Research Center of Kindai University Faculty of Medicine.

Immunohistochemistry. A procedure described previously was applied with minor modifications (14, 15). Briefly, surgical specimens were fixed with formalin and embedded in paraffin. The tissue samples were then cut into slices 7 μm thick. The tissue sections were then heated three times for 5 min in target-retrieval solution (DAKO, Kyoto, Japan) and stained with monoclonal antibody to total MET (Clone D1C2; Cell Signaling Technology, Danvers, MA, USA), monoclonal antibody to phospho-Tyr1234/1235 MET (Clone D26; Cell Signaling Technology) and normal rabbit IgG antibody (DAKO) followed by biotin-labeled goat anti-rabbit IgG antibody and Vectastain staining kit (Vector Laboratories, Burlingame, CA, USA). After the enzymatic development, sections were counterstained with Gill's hematoxylin. Images were obtained using a Biozero BZ-8000 fluorescence microscope (objective, ×40) and BZ-II Analyzer software (Keyence, Osaka, Japan). The software randomly selected five microscopic fields, and a positive result was given when MET-stained cells were observed more than 7% in each field. A negative result was given when the software defined that there were fewer than 7% stained cells.

Induction and culture of GSCs. The procedure has been described previously (12, 16). Briefly, GSCs were generated from the primary tumors of patients with GBM upon surgical removal. The fresh tumor specimens were directly digested with 0.05% trypsin into single cells and grown in neurobasal medium with 10% fetal bovine serum supplemented with epidermal growth factor (20 ng/ml; Gibco, Waltham, MA, USA), basic fibroblast growth factor (20 ng/ml; Gibco), and 1% penicillin and streptomycin (Gibco) at 37°C. Subsequently, 1×10^3 of the CD133-sorted MET-positive and MET-negative cells were plated out in triplicate and incubated for 5 days in 24-well cell culture plates.

Table I. *Diagnostic staging and histological profile of patients with glioma included in the study.*

	WHO grading		
Characteristic	II	III	IV
Number	11	7	95
Gender			
Male	10	4	49
Female	1	3	46
Age (median), years	42.3	39.9	61.5
MIB1 Index	N/A	N/A	23.1
OS (weeks)	294.7	231.6	57.1
Total-MET			
Positive	0	0	9
Negative	11	7	86

MIB1: Mindbomb E3 ubiquitin protein ligase 1, N/A: not available, OS: overall survival; Positive: defined as a frequency of MET-stained cells of more than 7%; negative: defined as a frequency of MET-stained cells fewer than 7%.

Flow cytometry. The procedure has been described previously with minor modifications (12, 17, 20, 22). Briefly, viable cells were stained with the following fluorescently labeled antibodies: anti-mouse CD133 (Clone 13A4; eBiosciences, San Diego, CA, USA) and isotype-matched control antibody (BD Biosciences, San Jose, CA, USA). Flow cytometric data were obtained using an Attune Acoustic Focusing Cytometer (Thermo Fisher Scientific, Waltham, MA, USA) and analyzed using WinList software (Verity Software House, Topsham, ME, USA). To sort CD133-positive cells, we used BD FACSAria (BD Biosciences).

Immunoblot analyses. A procedure described previously was applied with minor modifications (17-19). Briefly, GSCs were washed with phosphate-buffered saline (PBS) and lysed in CelLytic M (Sigma-Aldrich, Saint Louis, MO, USA) containing Protease Inhibitor Cocktail Complete (Roche Diagnostics, Mannheim, Germany) and Phosphatase Inhibitor Cocktail (Toyobo, Osaka, Japan). After 15 min incubation at room temperature, cell debris was removed by centrifugation. Cell lysates were electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel in reducing conditions and electrophoretically transferred to a polyvinylidene difluoride membrane. The membranes were blocked in 5% skim milk and probed with the following primary monoclonal antibodies: anti-MET (Clone D1C2), anti-phospho-Tyr1234/1235 MET (Clone D26), and α-tubulin (Clone 11H10; all Cell Signaling Technology). After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and developed using ECL Prime System (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Signal intensities were quantified using Image J software (NIH, Bethesda, MD, USA).

Intracranial tumor-bearing mouse model. The procedure for inoculating tumor cells into the brain of adult mice was described previously (17, 20, 21). Briefly, using a Hamilton syringe (Sigma-Aldrich), 1×10^3 GSCs were suspended in 2 μl PBS and stereotactically injected through an entry site at the bregma, 3 mm

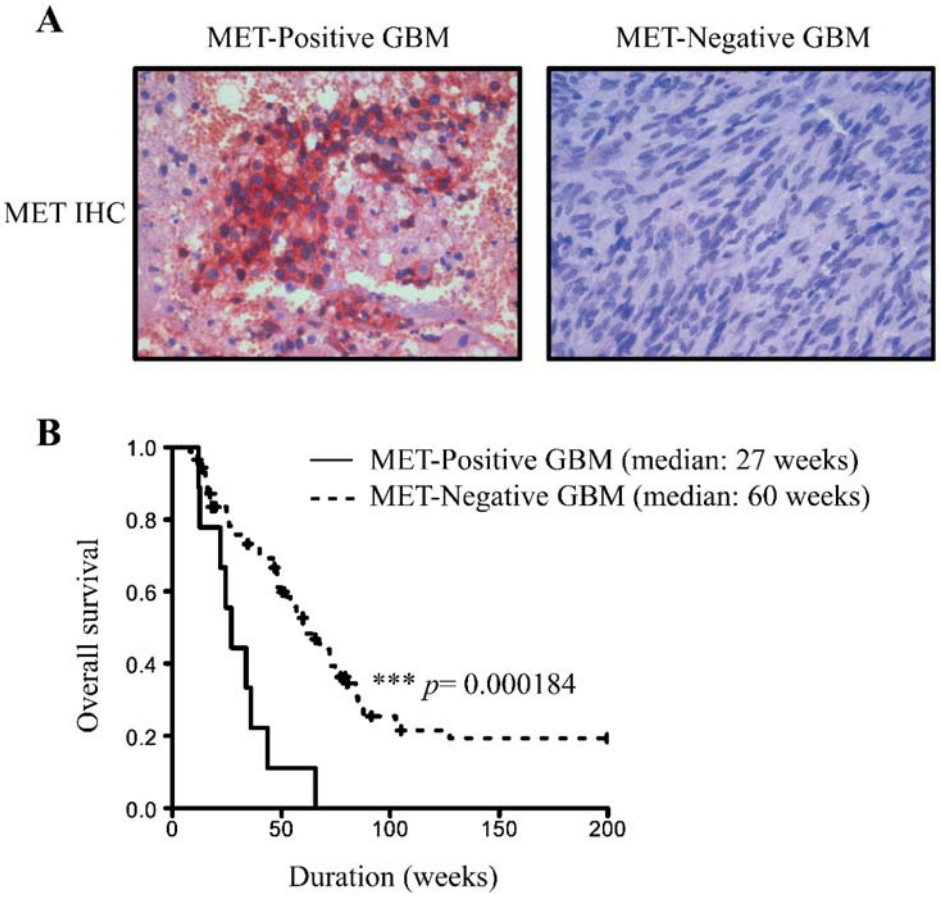

Figure 1. *Mesenchymal–epithelial transition (MET)-positive glioblastoma multiforme (GBM) is associated with poor survival. A: Representative images of MET-positive and MET-negative GBMs. B: Overall survival of patients with GBM was analyzed based on MET-expression status, with MET positivity being defined as a frequency of more than 7% of MET-stained cells; negative: defined as a frequency of MET-stained cells fewer than 7%. Log-rank test was performed to calculate p-values for the difference in survival.*

to the right of the sagittal suture and 4 mm below the surface of the skull of anesthetized mice (10 mice per group) using a stereotactic frame (Stolting Co., Wood Dale, IL, USA).

Crizotinib treatment in vitro and in vivo. The procedure has been described previously (19). Briefly, crizotinib was purchased from Wako Laboratory Chemicals (Tokyo, Japan) and dissolved in dimethyl sulfoxide (DMSO) for *in vitro* experiments and formulated in water for animal studies. In some experiments, GSCs were plated in 96-well plates and treated in triplicates with the following concentrations of crizotinib (1 μM, 10 μM, 100 μM, 1 mM, 10 mM, 100 mM, and 1 M), temozolomide (0.1 μM, 1 μM, 10 μM, 100 μM, 1 mM, 10 mM and 100 mM), or vehicle control (DMSO). Viability was assessed after 72 h of treatment using the Cell Counting Kit 8 (CCK8; Wako) according to the manufacturer's instructions. In other experiments, tumor-bearing mice (10 mice per group as described above) were given crizotinib at a dose of 50 mg/kg by oral gavage six times a week for 33 days. For the control group, 0.2 ml vehicle was given. The survival of tumor-bearing animals was recorded.

Statistical analyses. The procedure has been described previously (22). Briefly, Student's *t*-test was performed to analyze differences between two groups; log-rank test was performed to analyze survival. All data were analyzed using R Environment (R Development Core Team, Vienna, Austria) with EZR plugin version (23). A value of $p<0.05$ was considered to be statistically significant.

Results

Patients with MET-positive GBM exhibit poor survival. Among the available patients, we particularly focused on those with GBM in order to address the impact of MET on this type of tumor (Table I). The median age of patients with GBM at the time of diagnosis was 61.2 years [95% confidence interval (CI)=14-85 years]. There were 49 men and 46 women. The median MIB1 index was 23.1%. The mean follow-up at the time of analysis was 127 weeks (95% CI=31-562 weeks). The median overall survival (OS) was

78.8 weeks. At the end of follow-up, 28 (29.4%) patients remained alive.

We recorded nine patients who suffered from MET-positive GBM (Figure 1A). Among them, we recorded only one patient who had a phospho-MET-positive tumor (data not shown). Based on these data, we analyzed the survival of the patients according to their MET status. As a result, those with MET-positive GBM exhibited a poor OS (p=0.000184; Figure 1B) compared with those with MET-negative tumors.

MET-positive GSCs were established from human glioma tissues. Simultaneously, we collected the surgical specimens of GBMs with and without MET expression to establish spheroid-forming GSCs. To this end, we used culture conditions that have been established for isolation of neural stem cells (7). Within 24-48 h of primary culture, brain tumors yielded a minority fraction of cells that formed neurosphere-like clusters (tumor spheroids; Figure 2A), whereas the remaining populations of tumor cells were adherent (data not shown). The GSCs obtained from both MET-positive and MET-negative GBMS exhibited surface expression of CD133 at high levels (Figure 2B). We then sorted CD133-positive cells and cultured them. After several passage of the cells, we performed immunoblot analyses to detect MET protein (Figure 2C). We found the cells maintained their original properties regarding MET expression. Taken together, these data suggest that we were able to establish GSCs with and without MET expression.

MET-positive GSCs are susceptible to the MET inhibitor crizotinib. The results described above led us to hypothesize that MET-positive GSCs would be more susceptible to a MET inhibitor such as crizotinib than would MET-negative cells. To address this question, we treated the established GSCs with crizotinib or temozolomide (as a control) in a dose-escalating manner (Figure 3). The results showed crizotinib treatment effectively killed the MET-positive GSCs with an average half maximal inhibitory concentration (IC_{50}) of 87 μM (Figure 3A). In contrast, the MET-negative GSCs were found to be resistant to crizotinib with an average IC_{50} of 701 μM. In contrast, both MET-positive and MET-negative GSCs were resistant to temozolomide (Figure 3B). These data suggest the possibility of MET as a therapeutic target for GSCs.

Mice bearing MET-positive GSCs exhibited prolonged survival in response to crizotinib treatment. Subsequently, we hypothesized that MET-targeting therapy would also exhibit therapeutic efficacy against GBMs *in vivo*. To address this question, we established intracranial xenograft models using the human-derived GSCs and NOD-SCID mice and then treated the tumor-bearing mice with crizotinib (Figure 4). Both MET-positive and MET-negative GSCs formed brain tumor masses almost equally when the host mice were untreated (data not shown). When the mice bearing MET-positive GSCs were treated with crizotinib, they exhibited prolonged survival compared with those with control treatment (p=0.000833; Figure 4A). In contrast, the mice bearing MET-negative GSCs exhibited no prolongation of survival in response to crizotinib compared with control treatment (p=0.678; Figure 4B). Taken together, these data suggest that MET expressed in GSCs could be a potent therapeutic target for GBMs.

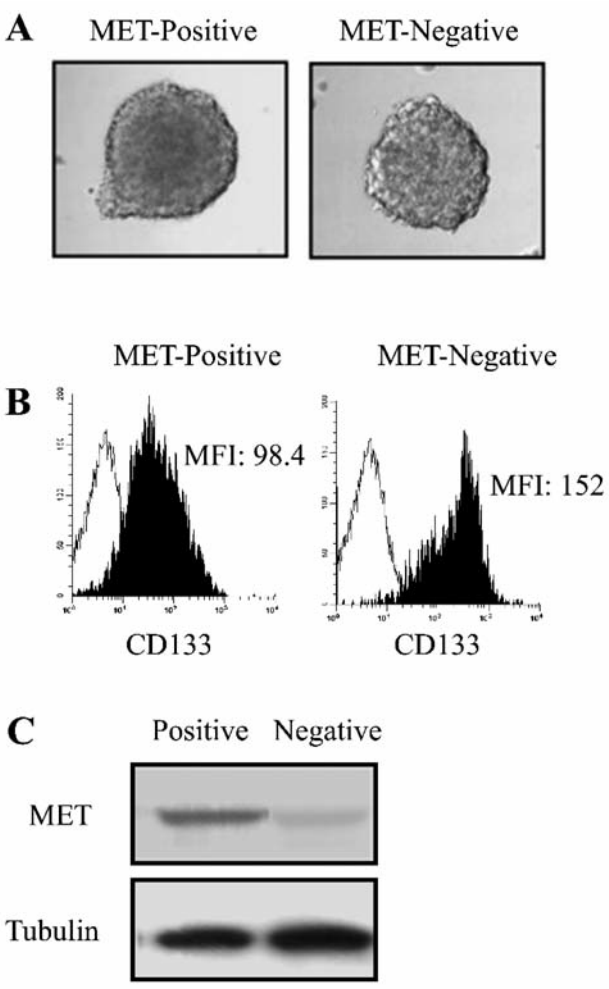

Figure 2. *Mesenchymal–epithelial transition (MET)-positive glioma stem cell-like cells (GSCs) were successfully established from human-derived glioblastoma multiforme (GBM) tissues. Spheroid-forming GSCs were established from the surgical specimens of GBMs with and without MET expression. A: Representative images of neurosphere formation by GSCs. B: Flow cytometry-based cluster of differentiation 133 (CD133) expression levels of GSCs. MFI: Mean fluorescent intensity. C: Immunoblot analyses for MET protein. α-Tubulin was used as an internal control.*

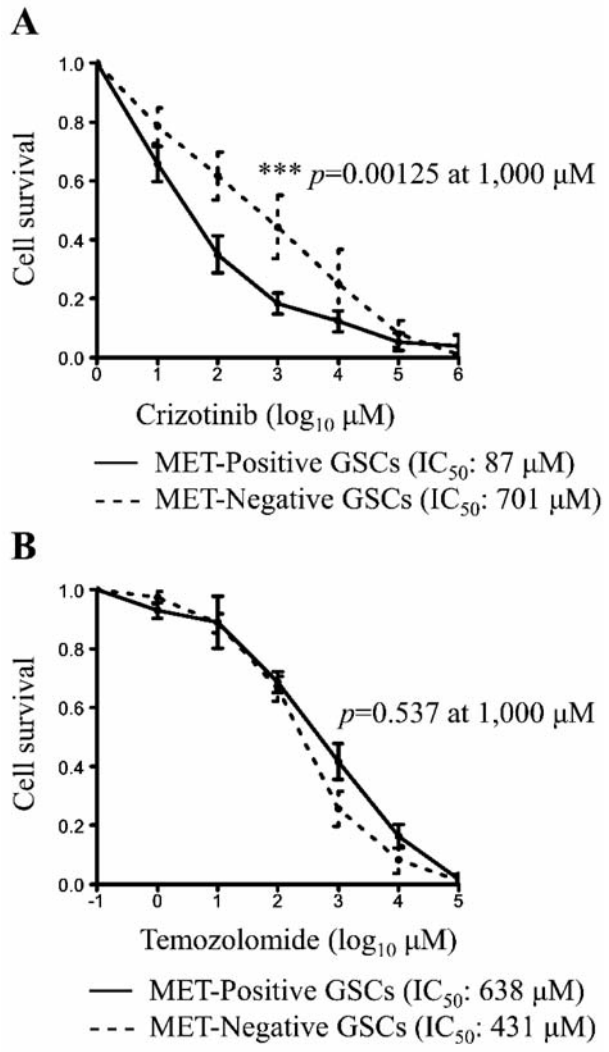

Figure 3. *Mesenchymal–epithelial transition (MET)-positive glioma stem cell-like cells (GSCs) are susceptible to the MET inhibitor crizotinib. Established MET-positive and MET-negative GSCs were treated with crizotinib (A) or temozolomide (B) for 48 h at the indicated concentrations in vitro. Cell viability was evaluated by the cell counting kit 8 assay. IC_{50}: Half-maximal inhibitory concentration. Student's t-test was performed to calculate p-values for differences between GSCs.*

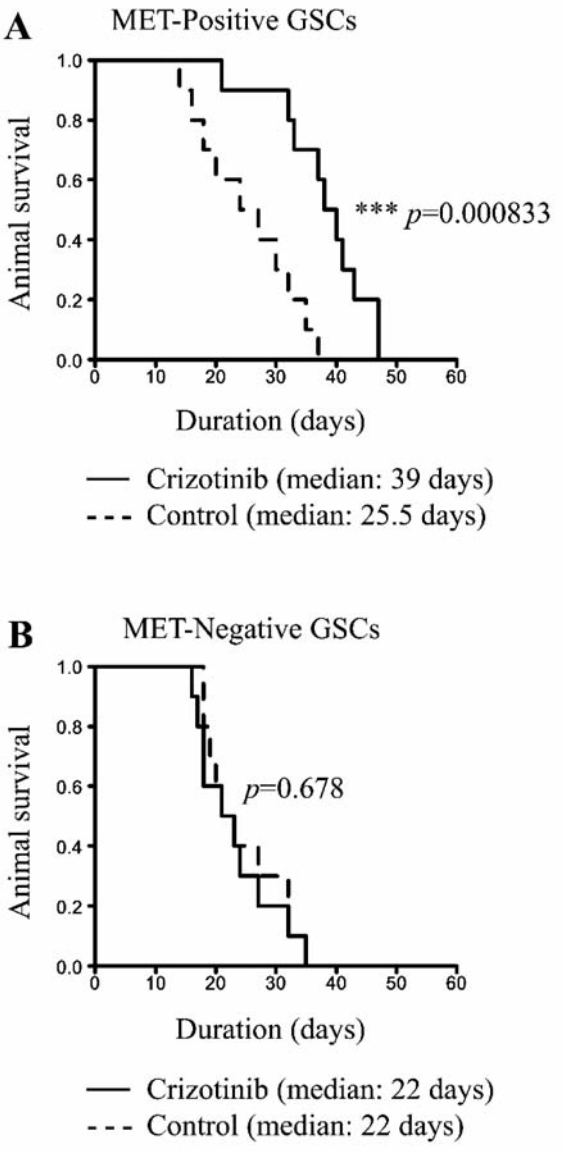

Figure 4. *Mice bearing mesenchymal–epithelial transition (MET)-positive glioma stem cell-like cells (GSCs) exhibited prolonged survival in response to crizotinib treatment. The GSCs inoculated into the brain of NOD-SCID mice formed brain tumor masses. The mice were treated with crizotinib or vehicle control from the beginning. Symptom-free survival of mice inoculated in the brain with MET-positive (A) and MET-negative (B) GSCs. Log-rank test was performed to calculate p-values for differences in survival.*

Discussion

In this study, we demonstrated the potency of MET-targeting therapy for GBM. In the first place, we analyzed our original cohort to determine whether MET expression status in GBM tumor tissues would correlate with the outcome of patients (Figure 1). As a result, the patients with MET-positive GBM had poor survival compared to those with MET-negative GBM. We succeeded in the establishment of both MET-positive and MET-negative GSCs (Figure 2). The GSCs were then subjected to crizotinib treatment both *in vitro* (Figure 3) and *in vivo* (Figure 4). These data revealed that MET-positive GSCs are more susceptible to crizotinib treatment than MET-negative GSCs. Taken together, our data suggest that MET expressed in GSCs could be a potent therapeutic target for GBMs.

The patients with MET-positive GBMs exhibited poor survival compared to those with MET-negative GBMs even without any MET-targeting therapy (Figure 1). This observation indicates that MET expression in tumor tissues would likely correlate with the malignancy of glioma cells, as suggested previously (4, 5). To address how MET is involved in the malignancy of glioma cells, we subsequently successfully established MET-positive and MET-negative GSCs (Figure 2-4). However, we have not performed *MET* gene knock-in or knock-down experiments using GSCs yet. To clarify the impact MET of expression on gliomagenesis, we need to conduct such experiments.

We fortunately had the opportunity to establish both MET-positive and MET-negative GSCs from tumor specimens (Figure 2). In a previous report, MET-positive GBM cells readily formed neurosphere-like aggregates and continued to proliferate, a fact that implies malignancy of the cells (24, 25). However, one of these studies demonstrated that MET-negative GBM failed to sustain active cell growth *in vitro* (24). In this regard, our MET-negative GSCs grew steadily (Figure 3). We speculate the reason for this difference to be as follows. In GSC growth, MET expression is not indispensable but sufficient. In addition, the assessment standard for cell growth might be different between their study and ours. Furthermore, the reproducibility of our methods for MET-negative GSCs might be stable. The next action plan for this research question is to repeat the induction of MET-negative GSCs and stabilize the procedure.

We also demonstrated that MET-positive GSCs are more susceptible to crizotinib treatment than MET-negative GSCs both *in vitro* (Figure 3) and *in vivo* (Figure 4). To address the impacts of MET expression on gliomagenesis, an animal model of spontaneous glioma would also be useful. In this regard, we have already developed such animal models using the Sleeping Beauty transposon system (12, 21). Since this system allows us to use any animal strain to induce spontaneous glioma formation *in vivo*, we can theoretically use genetically modified animals in which MET gene is either knocked-in or knocked-out. To our best knowledge, however, *MET* gene knockout in mice is embryonically lethal. Therefore, conditional *MET* knockout animals are widely used (26-28). Based on these findings, we are in the process of developing glial fibrillary acidic protein-driven *MET* gene knockout mice in which the *MET* gene is absent in gliomas in our spontaneous gliomagenesis model described above.

Collectively, we have demonstrated here the potency of MET-targeting therapy for GBM using MET-positive GSCs and crizotinib. However, we are aware that the inhibitory function of crizotinib is suboptimal for MET. Thus, the development of other MET inhibitors is mandatory. In this regard, several MET-specific inhibitors are currently in the process of clinical trials (29). In addition, given that MET is a cell-surface molecule, therapy using monoclonal antibody to MET may be applicable for this strategy. If we can efficiently inhibit MET that is expressed on GSCs, this might be a potent strategy for achieving complete protection against GBM recurrence.

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