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Aberrant methylation and silencing of the *SPINT2* gene in high-grade gliomas

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Hepatocyte growth factor activator inhibitor type 2 (HAI-2), encoded by the *SPINT2* gene, is a membrane-anchored protein that inhibits proteases involved in the activation of hepatocyte growth factor (HGF), a ligand of MET receptor. Epigenetic silencing of the *SPINT2* gene has been reported in a human glioblastoma cell line (U87) and glioblastoma-derived cancer stem cells. However, the incidence of *SPINT2* methylation in tumor tissues obtained from glioma patients is unknown. In this study, we analyzed the methylation status of the *SPINT2* gene of eight human glioblastoma cell lines and surgically resected glioma tissues of different grades (II, III, and IV) by bisulfite sequence analysis and methylation-specific PCR. Most glioblastoma lines (7/8) showed methylation of the *SPINT2* gene with a significantly reduced level of *SPINT2* mRNA compared to cultured astrocytes and normal brain tissues. However, all glioblastoma lines expressed mRNA for HGF activator (*HGFAC*), a target protease of HAI-2/SPINT2. Forced expression of *SPINT2* reduced MET phosphorylation of U87 glioblastoma cells both in vitro and in intracranial xenografts in nude mice. Methylation-specific PCR analysis of the resected glioma tissues indicated notable methylation of the *SPINT2* gene in 33.3% (2/6), 71.4% (10/14), and 74.3% (26/35) of grade II, III, and IV gliomas, respectively. Analysis of RNA sequencing data in a public database indicated an increased *HGFAC*/*SPINT2* expression ratio in high-grade compared to low-grade gliomas (P = .01). In summary, aberrant methylation of the *SPINT2* gene is frequently observed in high-grade gliomas and might confer MET signaling in the glioma cells.

KEYWORDS
gene methylation, glioma, HAI-2, HGF activator, SPINT2

1 | INTRODUCTION

Gliomas are the most common primary neoplasm in the central nervous system (CNS) and are classified based on the cellular lineages involved: astrocytomas, ependymomas, and oligodendrogliomas. These gliomas are further separated into low-grade (grades I and II) and high-grade tumors (grades III and IV) based on cell morphology, mitotic activities, and molecular markers. Glioblastoma is the most malignant form (ie, grade IV), whereas pilocytic astrocytoma is a benign localized-type glioma (grade I).[1,2] Glioblastoma is one of the most deadly forms of cancer in humans, with a median survival of 12-15 months and a 5-year survival rate of <5%.[2] Despite ongoing

research, including comprehensive genomic analyses, there has not been an improvement in the survival of patients suffering from glioblastoma.[1] The aggressive nature of glioblastoma is reflected by its extensive invasive growth. Intratumoral heterogeneity confers malignant phenotypes on glioblastoma. This heterogeneity is caused by mixed gene mutations, including amplification, and by regional differences in gene expression.[3]

Hepatocyte growth factor activator inhibitor (HAI) is a type I transmembrane serine protease inhibitor. At present, two HAIs are known: HAI-1 (encoded by the *SPINT1* gene) and HAI-2 (*SPINT2*). Both have two extracellular Kunitz-type serine protease inhibitor domains, a transmembrane domain, and a C-terminal short intracytoplasmic domain, and they are expressed in most epithelial tissues and the placenta.[4] However, HAI-2/serine peptidase inhibitor, Kunitz type 2 (SPINT2) is preferentially expressed in CNS tissues, and in murine CNS, HAI-2/SPINT2 is highly expressed in the glomerular layer of the olfactory bulb, in the cerebral cortex, and in the striatum, whereas HAI-1/SPINT1 is hardly detectable.[5] Both HAIs were initially identified as endogenous cellular inhibitors of hepatocyte growth factor activator (HGFA).[6,7] However, it is now well known that HAIs also regulate other cellular proteases responsible for the activation of the proform of hepatocyte growth factor (HGF), such as matriptase and hepsin.[4,8] The extracellular activation of proHGF is critical for the biological activity of HGF through its specific receptor tyrosine kinase (MET); MET-induced signals are involved in invasive growth and drug resistance of various types of cancers.[4,9] Therefore, HAI-1/SPINT1 and HAI-2/SPINT2 have generally been implicated as suppressors of cancer progression by inhibiting HGF-MET signal transduction,[4] although contradicting results have also been reported for HAI-2/SPINT2.[10-12]

Previously, we reported that *SPINT2* mRNA levels were significantly reduced along with the progression of gliomas and HAI-2/SPINT2 protein suppressed Matrigel invasion of glioblastoma cell lines.[13] Subsequently, it was reported that the hypermethylation of the *SPINT2* promoter region underpins reduced HAI-2/SPINT2 expression in the U87 human glioblastoma cell line.[14] Furthermore, methylation of the *SPINT2* gene was also reported in glioblastoma-derived cancer stem cells.[15] Medulloblastoma, another highly malignant CNS tumor, also shows hypermethylation and silencing of the *SPINT2* gene.[16,17] However, the methylation status of the *SPINT2* gene in human glioma tissues and its relationship to glioma progression have not yet been clearly elucidated. In this study, we aimed to analyze methylation of the 5′-CpG island of the *SPINT2* gene in a series of human glioblastoma cell lines and surgically resected glioma tissues of different histopathological grades (grades II-IV). We also analyzed the effect of the forced expression of HAI-2/SPINT2 on the growth and MET phosphorylation of glioblastoma cell lines.

2 | MATERIALS AND METHODS

2.1 | Cell culture and human tissue samples

The human glioblastoma cell lines U251, YKG1, A172, T98G, KS1, U87, YH13, and NYGM were maintained in DMEM containing 10% FBS. U251, YKG1, T98G, A172, and KS1 were obtained from the Riken Cell Bank (Tsukuba, Japan). NYGM was established in our laboratory.[18] YH13 was from the Health Science Research Resource Bank (Osaka, Japan). U87 was from ATCC through Dainippon Sumitomo Pharma (Osaka, Japan). Immortalized human astrocyte cell line (T0281) was purchased from Applied Biological Materials (Vancouver, Canada) and maintained using Prigrow IV medium and PriCoat T25 flasks (Applied Biological Materials).

The experimental protocol used to obtain clinical samples was approved by the Human Ethics Review Committees of Miyazaki University (Miyazaki, Japan) (approval number 2014-023). Tissue samples were obtained from surgically resected low-grade gliomas, anaplastic gliomas, and glioblastomas with written informed consent from each patient. The histological diagnosis and grading of tumors were assessed according to the WHO classification. Normal genomic DNA from the whole brain of a fetus and an adult was purchased from BioChain Institute (Newark, CA, USA) and Epigentek Group (Farmingdale, NY, USA), respectively. Total RNA from a normal brain was obtained from Takara Bio (Shiga, Japan).

2.2 | Reverse transcription-PCR and immunoblotting

Reverse transcription-PCR was carried out as described elsewhere using the following primers:[12] *SPINT2*, forward 5′-CGGGGCAATAAGAACAGCT-3′ and reverse 5′-AGCTGCTCCTTGTCATCATCTCC-3′; and β-actin (*ACTB*), forward 5′- ATTGCCGACAGGATGCAGA -3′ and reverse 5′- GAGTACTTGCGCTCAGGAGGA -3′. Primer sequences for HGFA (*HGFAC*), matriptase (*ST14*), hepsin (*HPN*), *TMPRSS2*, *TMPRSS13*, human airway trypsin-like protease (HAT: *TMPRSS11D*), *HGF*, *MET*, HAI-1 (*SPINT1*), and *GAPDH* are described in Table S1.

For immunoblotting, cultured cells (approximately 70% confluency) were washed 3 times with PBS and the cellular proteins were extracted with 1% (v/v) Triton X-100 in PBS with protease inhibitor cocktail (Merck & Co., Kenilworth, NJ, USA). For immunoblotting, each extracted protein was separated by SDS-PAGE under nonreducing conditions, transferred onto an Immobilon membrane (Millipore, Bedford, MA, USA), and processed for HAI-2/SPINT2 detection using mouse mAb 2A6121 as described elsewhere.[12] For the detection of MET and phosphorylated MET, cells were extracted in the presence of 100 mmol/L NaF and 1 mmol/L Na_3VO_4 and SDS-PAGE was carried out under reducing conditions. Anti-human MET mouse mAb was kindly provided by Dr. D. Naka, Yokohama Research Center, Mitsubishi Pharma (Yokohama, Japan), and antiphosphorylated (Tyr1234/1235) MET rabbit mAb was purchased from Cell Signaling Technology (Boston, MA, USA).

2.3 | Bisulfite modification and methylation analysis

The promoter region of the *SPINT2* gene was identified with the human genome browser (http://genome.ucsc.edu), and the CpG island around the putative promoter region was predicted by Methyl Primer Express Software (Applied Biosystems Japan, Tokyo, Japan).

One microgram of genomic DNA was subjected to sodium bisulfite conversion using an EpiTect Bisulfite Kit (Qiagen, Tokyo, Japan), and 1/20 of the converted DNA was used for each PCR. For bisulfite sequence analysis, the *SPINT2* promoter region was amplified with PCR using DNA with bisulfite conversion as a template, using the following primers: forward 5′-TAAGTTTAAGGGAAGGGTGGTA-3′ and reverse 5′-TACCTAAATCTACTCCTCACTC-3′. The PCR products were subcloned into plasmids using a TOPO TA Cloning Kit (Life Technologies, Tokyo, Japan). The plasmid DNA from isolated colonies of transformed *Escherichia coli* was extracted, and DNA from multiple independent clones was sequenced to determine *SPINT2* methylation status. A Web-based tool, QUMA (http://quma.cdb.riken.jp) was used for the visualization of bisulfite sequence data.[19]

For methylation-specific PCR (MSP), published primers designed to amplify either the unmethylated or methylated *SPINT2* promoter region DNA after bisulfite conversion were used.[20] The primer sequences are as follows: unmethylated DNA, forward 5′-GGTTGGGTGTTTTTATATTGAAGGTTT-3′ and reverse 5′-TCAACACCACCAACCATTAAAATCTCA-3′; and methylated DNA, forward 5′-CGGGCGTTTTTATATTGAAGGTTC-3′ and reverse 5′-ACGCCACCAACCGTTAAAATCTCG-3′. The annealing temperatures for unmethylated and methylated DNAs were 58°C and 54°C, respectively, for 30 seconds. Hot start PCR with a total of 32 cycle numbers was applied for the amplification. The reaction products were separated by electrophoresis on 5%-12% gradient PAGE and visualized by ethidium bromide. The mean signal intensity and the total pixel number of each band image were measured by Photoshop software (Adobe Systems, San Jose, CA, USA) to calculate the signal level. Treatment of cultured cells with 5-azacytidine (Sigma-Aldrich, St. Louis, MO, USA) was carried out in accordance with the method described.[21]

2.4 | Forced expression of HAI-2/SPINT2 and cell proliferation assay

Generation of the HAI-2/SPINT2 expression plasmid was described previously.[13] Briefly, the full-length coding region for HAI-2/SPINT2 was cloned into *Xba*I-*Sal*I sites of a pCI-neo expression vector (Promega, Madison, WI, USA). An empty vector (mock) or *SPINT2* expression plasmid was linearized and transfected into U87, U251, and T98G to establish clones stably expressing HAI-2/SPINT2. To determine growth curves of the mock- or *SPINT2*-transfected subline, triplicate 35-mm dishes were seeded at 1×10^4 cells/3 mL growth medium and the number of viable cells was counted at the indicated time period.

2.5 | Intracranial implantation of glioblastoma cells in nude mice

All animal procedures were undertaken in accordance with institutional guidelines, and the protocol was approved by the Animal Care Committee of the University of Miyazaki. Six-week-old male athymic nude mice (BALB/cAJc1-nu; mean body weight, 20 g) were obtained from CLEA Japan (Tokyo, Japan). For intracranial transplantation, 1×10^5 U87 cells with or without forced expression of *SPINT2* were suspended in 10 μL PBS and stereotactically transplanted into the forebrain of mice as described previously.[20,22] The mice were carefully observed every day for 98 days to calculate the Kaplan-Meier survival curves. Brain specimens were prepared after euthanasia from each dying mouse and from mice 98 days after the transplantation. The brain tissues were fixed with 4% formaldehyde in PBS and sectioned coronally at the point of cellular implantation, followed by embedding in paraffin.

2.6 | Immunohistochemistry

Formalin-fixed paraffin-embedded tissue sections (4 μm) were processed for immunohistochemistry. The staining was carried out on the Leica Bond-Max III automated immunostainer (Leica Biosystems, Tokyo, Japan) according to the manufacturer's instructions. Heat treatment for antigen retrieval lasted for 30 minutes. The primary antibody used for immunohistochemistry was antiphosphorylated MET (Tyr1235) rabbit polyclonal antibody (0.5 μg/mL) reported previously.[23] To evaluate the immunoreactivity of phosphorylated MET, we graded the intensity of immunoreactivity (3, easily recognizable the cell surface immunoreactivity with a 4× objective lens; 2, recognizable cellular immunoreactivity with a 4× objective lens; 1, recognizable cellular immunoreactivity with a 200× objective lens; and 0, no immunoreactivity) and positive ratio in a hot spot (2, positive in ≥50% of tumor cells with a 10× objective lens; 1, positive in ≥10% and <50%; and 0, positive in <10%), and total score intensity grade + positive ratio grade was designated as the phosphorylated MET score.

2.7 | The Cancer Genome Atlas data collection and analysis

SPINT2 RNA sequencing (RNA-Seq) expression data, available for gliomas, were retrieved from The Cancer Genome Atlas (TCGA) Research Network (http://cancergenome.nih.gov/). Data were extracted from TCGA through the cBioPortal for Cancer Genomics website (http://www.cbioportal.org/) on November 17, 2016. In total, we selected 320 grade II gliomas, 206 grade III gliomas (anaplastic astrocytomas/oligodendrogliomas), and 160 grade IV gliomas (glioblastomas).

2.8 | Statistical analysis

Data were analyzed with R (The R Foundation for Statistical Computing, Vienna, Austria) using EZR software (Saitama Medical Center, Jichi Medical University, Saitama, Japan) that has a graphical user interface for R.[24] Comparison between two unpaired groups was made with the Mann-Whitney *U* test or two-way repeated-measures ANOVA. The χ^2 test was used for assessment of the relationship

between variables. Survival analysis was assessed by estimating the Kaplan-Meier survival curves, which were compared by log-rank test. Significance was set at $P < .05$.

3 | RESULTS

3.1 | Silencing of the *SPINT2* gene in glioblastoma cell lines by hypermethylation of 5′ CpG island

Initially, we analyzed the expression of HAI-2/SPINT2 in eight glioblastoma cell lines, an immortalized astrocyte cell line, and adult brain tissue. Consistent with a previous study,[13] *SPINT2* mRNA was hardly detected in any of the glioblastoma cell lines except for YH13 (Figure 1A). Consequently, a notable level of HAI-2/SPINT2 protein was detectable only in extracts from YH13 cells (Figure 1A). In contrast, astrocytes and adult brain tissue expressed substantial levels of *SPINT2* mRNA (Figure 1A). We also examined the expression of genes for presumed target proteases of HAI-2/SPINT2, all of which are known to activate proHGF.[4] Of note, low but distinct levels of *HGFAC* mRNA that encodes HGFA were consistently expressed by the glioblastoma cells (Figure 1B). The mRNAs for other proHGF-activating proteases were hardly detectable. We then examined the methylation status of a 5′-CpG island (Figure 1C) of the *SPINT2* gene. The results of both MSP (Figure 1D) and bisulfite sequencing (Figure 1E) were consistent with the *SPINT2* expression status observed by RT-PCR analysis, suggesting that HAI-2/SPINT2 is epigenetically downregulated in glioblastoma cell lines by hypermethylation of the *SPINT2* gene. Indeed, treatment of the glioblastoma cells with 5-azacytidine restored the expression of *SPINT2* mRNA (Figure S1).

3.2 | Forced expression of HAI-2/SPINT2 suppressed proliferation of glioblastoma cells

Previously, we reported that transient overexpression of *SPINT2* suppressed Matrigel invasion of U251 and YKG1 glioblastoma cell lines.[13] Moreover, a recent study indicated that the U87 glioblastoma cell line stably overexpressing *SPINT2* showed reduced cell growth and anchorage-independent colony formation.[15] In this study, we used U87, U251, and T98G cells to test the effect of forced *SPINT2* expression on the growth in vitro. In all cell lines, stable transfection of the *SPINT2* expression vector resulted in overexpression of HAI-2/SPINT2 protein (Figure 2A) and significantly reduced the growth rate (Figure 2B). Then, we analyzed the effects of HAI-2/SPINT2 on tumorigenicity of U87 cells in vivo by intracranial implantation. The tumorigenicity rates during the observation period (98 days) were 57.1% (8/14) and 46.7% (7/15) for mock-transfected U87 and *SPINT2* expression vector-transfected U87 cells, respectively. The forced *SPINT2* expression alleviated the mortality, as observed in the Kaplan-Meier survival curves of the implanted mice, although the difference was not statistically significant as determined with a log-rank test (Figure 2C).

3.3 | HAI-2/SPINT2 reduced MET phosphorylation of U87 cells in vitro and in vivo

To study the role of HAI-2/SPINT2 in HGF-MET signaling in glioblastoma, we asked whether the forced expression of HAI-2/SPINT2 altered MET activation in vitro and in tumors generated by intracranial implantation of U87 cells in vivo. This cell line reportedly has an HGF-MET autocrine loop,[25] and we confirmed concomitant expression of HGF and MET in this cell line (Figure 3A). The activation of MET was verified by an antibody that specifically recognized phosphorylated (Tyr1234/1235) MET. Immunoblot analysis showed that HAI-2/SPINT2 reduced the phosphorylation of MET in cultured U87 cells in vitro (Figure 3B). For the analysis of in vivo tumors, we undertook immunohistochemical staining using antiphosphorylated MET antibody.[23] The intracranial tumor tissues generated by mock-transfected U87 (n = 7) and *SPINT2* vector-transfected U87 (n = 7) cells were processed for immunohistochemistry. As shown in Figure 3C,D, forced expression of HAI-2/SPINT2 protein significantly reduced MET phosphorylation in vivo.

3.4 | *SPINT2* is highly methylated in high-grade glioma tissues

Whereas methylation of the *SPINT2* gene has been reported in cultured glioblastoma cells, its incidence in human glioma tissues in vivo is unknown. Therefore, we analyzed the methylation status of *SPINT2* in 56 cases of resected human glioma tissues, consisting of 6 cases of grade II, 14 cases of grade III, and 36 cases of grade IV tumors, by MSP. An easily visible methylation-specific PCR band with a signal level at least more than 10% of the corresponding nonmethylated PCR product was judged as methylation-positive in this analysis. Representative results of MSP are shown in Figure 4, and the whole data regarding the *SPINT2* methylation status with some genetic characteristics (*MGMT* gene methylation status, *TERT* gene promoter mutation, and *IDH1* gene mutation) are indicated in Table S2. Informative signals were obtained from all cases except for one glioblastoma patient (IV-17; Table S2) who was excluded from the following analyses. Methylation of the *SPINT2* gene was observed in 33.3% (2/6), 71.4% (10/14), and 74.3% (26/35) of grade II, grade III, and grade IV tumors, respectively (Table 1). Therefore, the *SPINT2* gene is highly methylated in high-grade gliomas (grades III and IV). Unmethylated specific PCR products were also amplified in most high-grade gliomas that were methylated. This result was not unexpected as there was contamination of non-neoplastic cells in the collected tissue samples.

3.5 | Increased *HGFAC*/*SPINT2* mRNA ratio in high-grade gliomas

We determined the relationship between the methylation status and overall survival of patients after surgery. *SPINT2* methylation status was not associated with the patients' survival in this study (Figure S2). A significant relationship was also not observed between *SPINT2* methylation and *MGMT* methylation or *TERT* mutation status (χ^2 test).

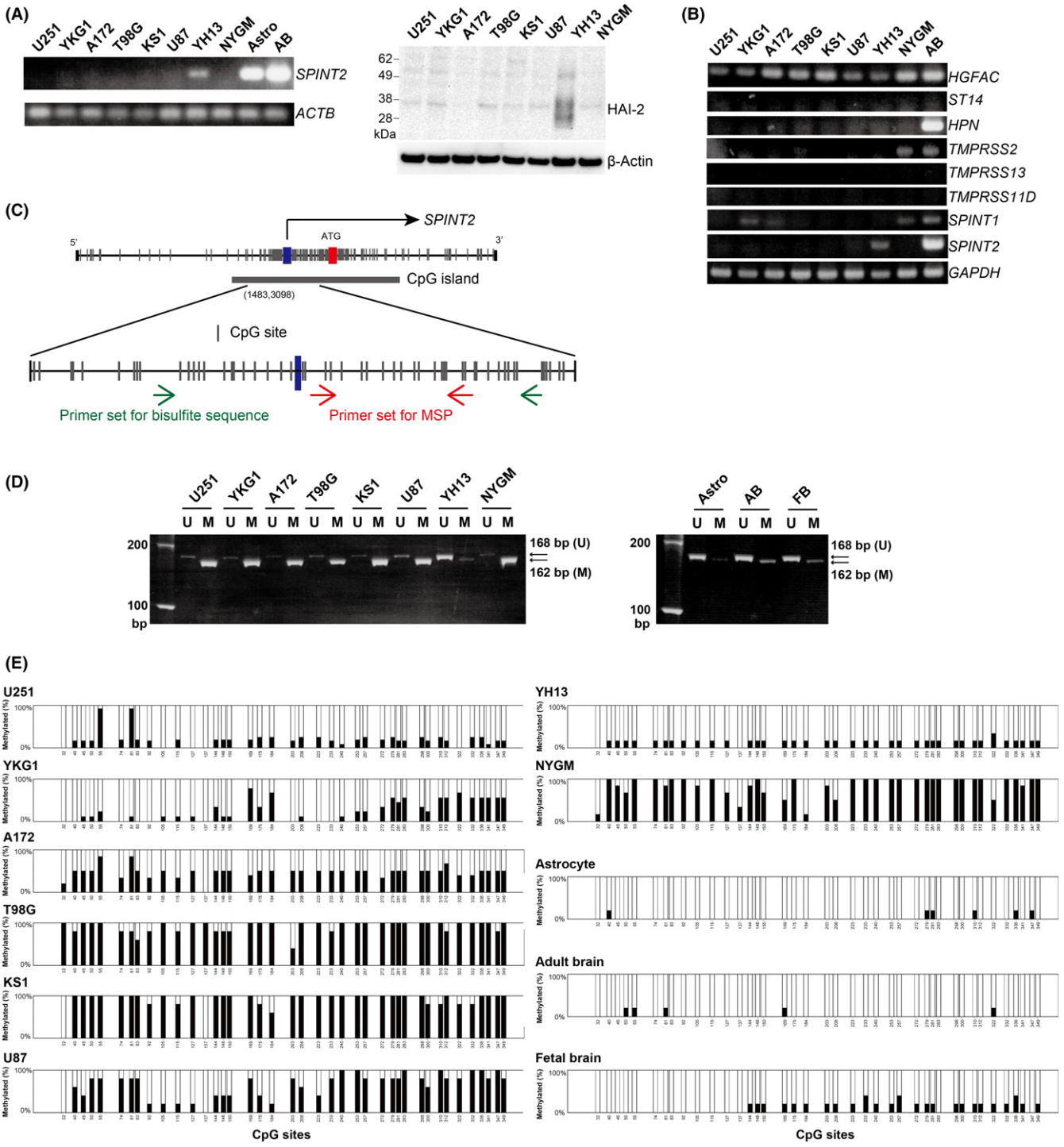

FIGURE 1 Expression and methylation status of *SPINT2* in human glioblastoma cell lines. A, RT-PCR analysis of *SPINT2* mRNA (left panel) and immunoblot analysis of hepatocyte growth factor activator inhibitor type 2 (HAI-2)/serine peptidase inhibitor, Kunitz type 2 (SPINT2) protein in glioblastoma cells. AB, adult brain; Astro, astrocytes. B, RT-PCR analysis of mRNAs for HAI-2-target proteases: HGFA (*HGFAC*), matriptase (*ST14*), hepsin (*HPN*), TMPRSS2 (*TMPRSS2*), TMPRSS13 (*TMPRSS13*), and HAT (*TMPRSS11D*). RT-PCR data for HAI-1/SPINT1 (*SPINT1*) and HAI-2/SPINT2 (*SPINT2*) are also shown. AB, adult brain. C, Presumed CpG island in the promoter region and exon 1 of the *SPINT2* gene. Positions of the primers for methylation-specific PCR (MSP; red arrows) and bisulfite sequence (green arrows) are also indicated. D, MSP analysis for the *SPINT2* gene of glioblastoma cell lines, immortalized human astrocyte cell line (Astro), normal adult brain tissue (AB), and fetal brain tissue (FB). Positions of unmethylated (U; 168 bp) and methylated (M; 162 bp) bands are indicated by arrows. E, Results of bisulfite sequencing. Methylation status of 40 CpG dinucleotides around the transcription start site of the *SPINT2* gene. Values are % of methylated clone of 10 (U251), 9 (YKG1), or 5 (others) clones analyzed

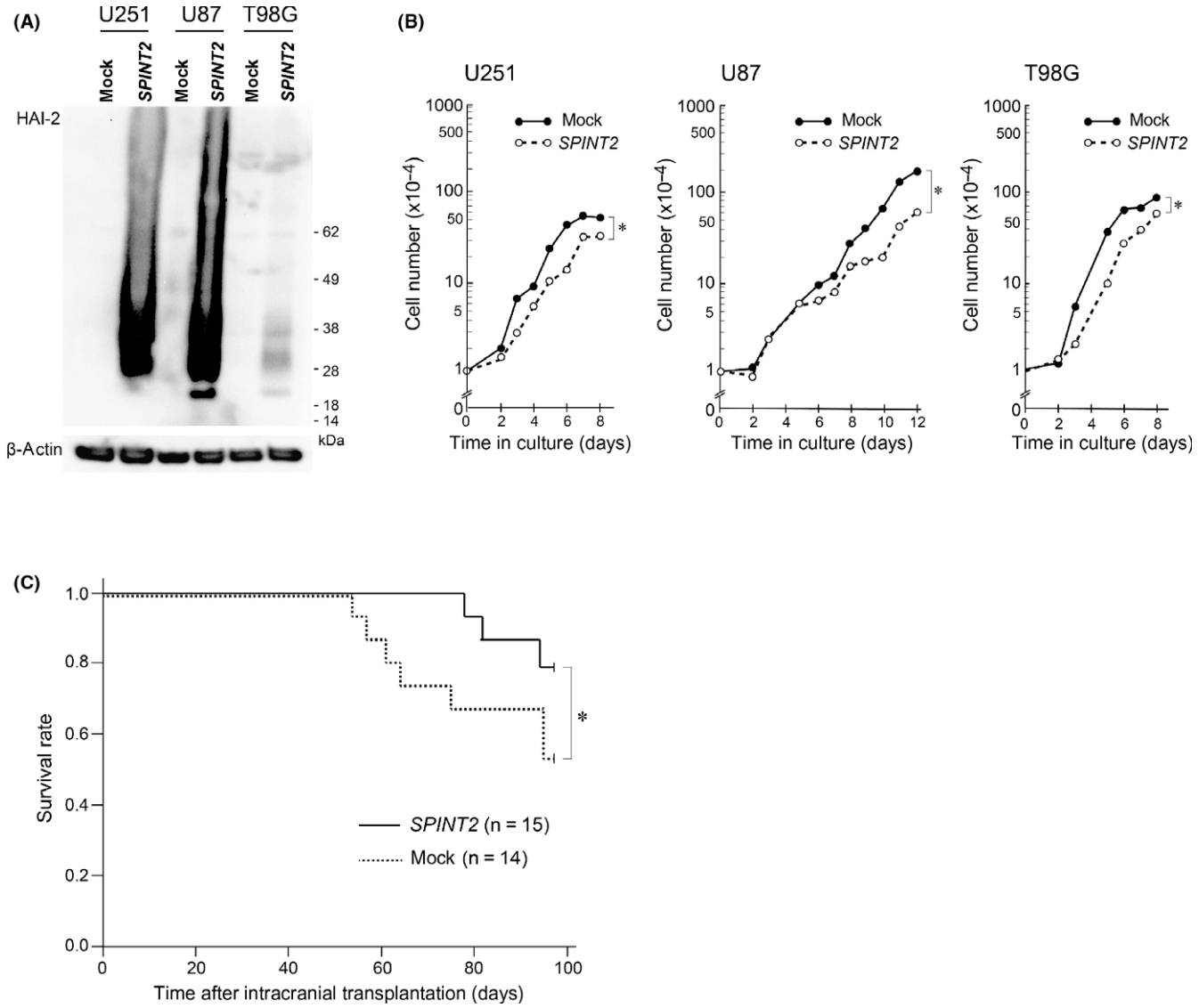

FIGURE 2 Effects of forced *SPINT2* expression on proliferation of glioblastoma cells in vitro and in intracranial transplantation in nude mice. A, Immunoblot analysis for hepatocyte growth factor activator inhibitor type 2 (HAI-2)/serine peptidase inhibitor, Kunitz type 2 (SPINT2) of cellular extracts from mock-transfected (mock) or *SPINT2*-transfected (*SPINT2*) glioblastoma cells (U251, U87, and T98G). B, Reduced cellular growth in vitro by forced expression of *SPINT2* in glioblastoma cell lines. The growth curve is shown on a semilogarithmic graph. $^*P < .01$ compared to mock (two-way ANOVA). C, Kaplan-Meier survival curves of mice after intracranial transplantation of U87 glioblastoma cell line transfected with (*SPINT2*) or without (mock) *SPINT2* expression vector. $^*P = .135$ (log-rank test)

However, most cases of *MGMT*-methylated high-grade gliomas showed *SPINT2* methylation (14/16) (Table 2). It is reasonable to speculate that the biological role of protease inhibitors might depend on the level of its target protease. Among target proteases of HAI-2/SPINT2, only HGFA was consistently expressed in cultured glioblastoma cell lines (Figure 1B). Therefore, we asked whether the expression ratio of *HGFAC* to *SPINT2* (*HGFAC*:*SPINT2*) was increased along with glioma progression. For this purpose, we undertook a gene expression analysis using RNA-Seq data in TCGA public database. The *HGFAC* RNA-Seq tended to increase along with glioma progression, whereas *SPINT2* RNA-Seq decreased in high-grade gliomas compared to low-grade gliomas (Figure 5A). As a result, *HGFAC*:*SPINT2* was increased in high-grade gliomas (n = 366) compared to low-grade gliomas (n = 320) at a statistically significant level ($P = .039$) (Figure 5B). In addition, although a higher *HGFAC*:*SPINT2* ratio did not show significant impact on the overall survival of patients suffering from high-grade glioma ($P = .105$, log-rank test) (Figure 5C, left panel), there was a relationship between higher *HGFAC* expression and shorter overall survival ($P = .010$) (Figure 5C, right panel). In contrast, the *SPINT2* RNA-Seq level was not associated with overall survival of high-grade glioma patients ($P = .773$, log-rank test).

4 | DISCUSSION

In this study, we analyzed the methylation status of the 5′-CpG island of the *SPINT2* gene in a series of human glioblastoma cell lines and

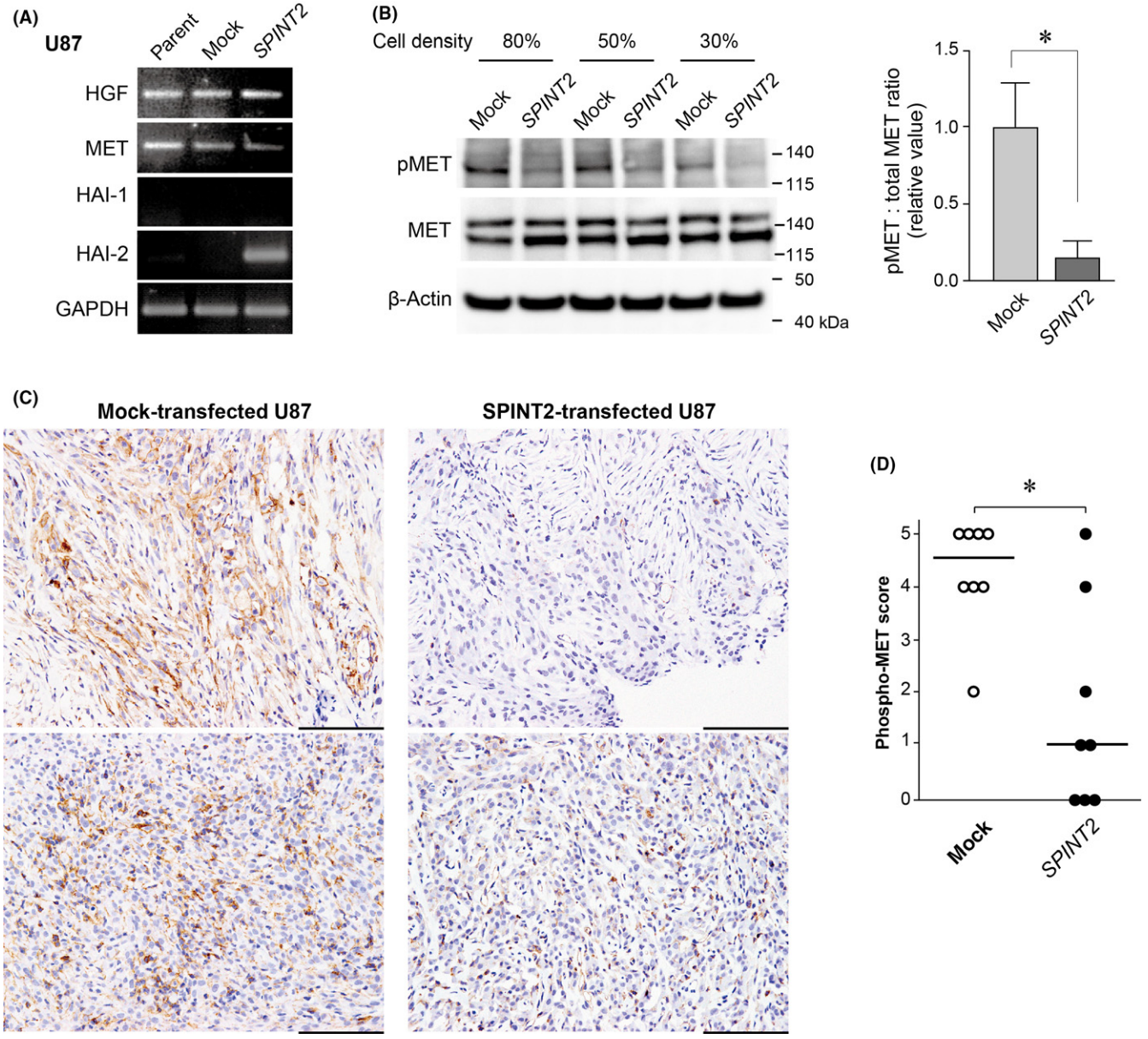

FIGURE 3 Decreased phosphorylation of MET in response to hepatocyte growth factor activator inhibitor type 2 (HAI-2)/SPINT2 expression in U87 glioblastoma cells. A, Expression of hepatocyte growth factor (HGF), MET, HAI-1, and HAI-2 in the cultured U87 glioblastoma cell line and its mock-transfected (mock) and *SPINT2* expression vector-transfected (*SPINT2*) sublines. B, Effect of HAI-2/serine peptidase inhibitor, Kunitz type 2 (SPINT2) expression on MET phosphorylation in U87 cells. Immunoblot data of three independent experiments using different culture densities are shown in the left panel. pMET, phosphorylated (Tyr1234/1235) MET. The band signal of pMET relative to that of corresponding total MET at 80% culture density was calculated and indicated in the right panel. Values are mean ± SD of triplicate experiments. *P = .008 (Student's t test). C, Immunohistochemistry of phosphorylated (Tyr1235) MET of intracranial tumors of mock- or *SPINT2*-transfected U87 cells. Representative images from two independent tumors of each subline are shown. Bar = 100 μm. D, Immunostaining score for phosphorylated MET. *P = .013 (Mann-Whitney U test). Bar, median

surgically resected glioma tissues. In accordance with previous reports using U87 cells,[14,15] most glioblastoma cell lines showed methylation of the *SPINT2* gene, and that reversion of *SPINT2* expression in glioblastoma cell lines (U87, U251, and T98G) suppressed the growth rate in vitro. Moreover, forced expression of HAI-2/SPINT2 reduced MET phosphorylation of U87 cells both in vitro and in vivo. The U87 cell line is known to express proHGF and MET concomitantly to establish an autocrine HGF-MET signaling loop.[25] Therefore, HAI-2/SPINT2 might suppress HGF-MET signaling by inhibiting the proHGF-activating protease. Several serine proteases are known to activate proHGF in the pericellular microenvironment: HGFA (*HGFAC*), matriptase (*ST14*), hepsin (*HPN*), TMPRSS2, TMPRSS13, and HAT (*TMPRSS11D*).[4] Among these proteases, only *HGFAC* mRNA was consistently detected in most glioblastoma cell lines in vitro with the sensitive RT-PCR method used in this study. Whereas HGFA is mainly produced by the liver and circulates in plasma as its zymogen form,

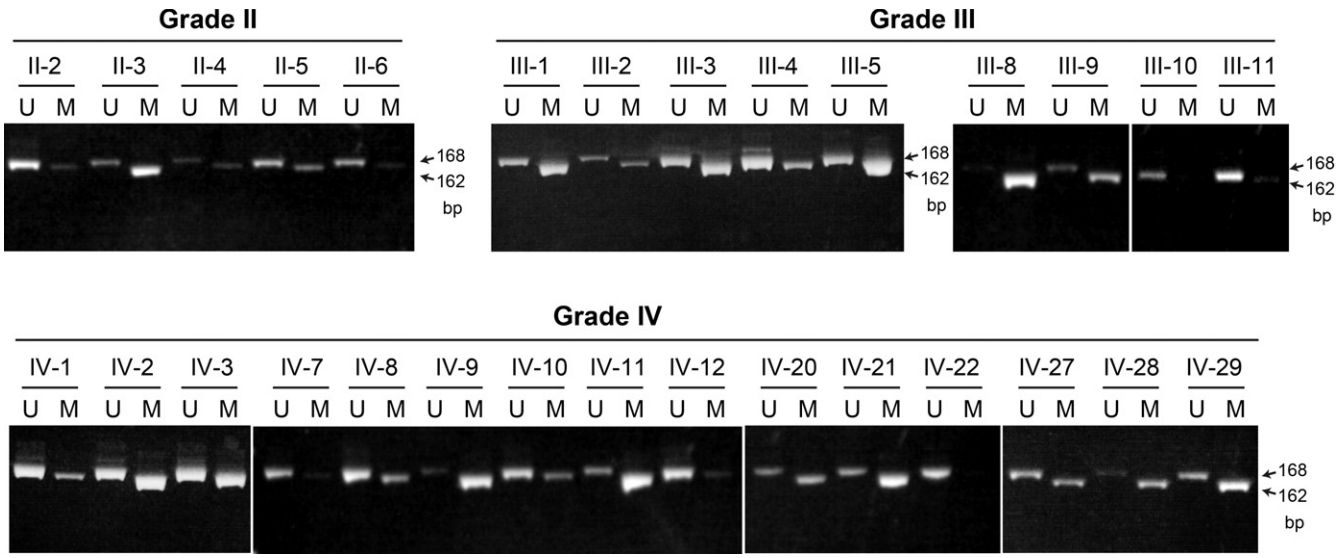

FIGURE 4 Results of methylation-specific PCR analysis of *SPINT2* in human glioma tissues. Representative data of grade II, III, and IV gliomas are shown. Positions of unmethylated (U; 168 bp) and methylated (M; 162 bp) bands are indicated by arrows

TABLE 1 *SPINT2* methylation status in glioma tissues

Grade	Pathological diagnosis	*SPINT2* methylation	
II	Diffuse astrocytoma	2/5	2/6 (33.3%)
	Oligodendroglioma	0/1	
III	Anaplastic astrocytoma	6/10	10/14 (71.4%)
	Anaplastic oligodendroglioma	4/4	
IV	Glioblastoma	25/34	26/35 (74.3%)
	Diffuse midline glioma	1/1	

TABLE 2 Correlation between *SPINT2* methylation status and *MGMT* methylation status in high-grade gliomas (grades III and IV)

	MGMT unmethylated	*MGMT* methylated
SPINT2 unmethylated	11 (8)	2 (1)
SPINT2 methylated	22 (15)	14 (11)

Numbers in parentheses represent the number of grade IV glioma cases. $P = .121$ for grade III + IV gliomas; $P = .089$ for grade IV gliomas (χ^2 test).

extrahepatic expression of *HGFAC* mRNA has also been reported and *HGFAC* mRNA was detected in 4 of 5 high-grade glioma tissues.[26,27] Therefore, we assume that both circulating and tumor cell-derived HGFA might be responsible for the activation of proHGF in human glioblastoma tissues. In fact, a previous report revealed that HGFA enhances MET phosphorylation of glioblastoma cells in vivo.[21] Our current analysis of TCGA database also supports this hypothesis, showing an increased *HGFAC*:*SPINT2* RNA-Seq ratio in high-grade gliomas compared to low-grade ones. Interestingly, a recent study suggested that epigenetic silencing of *SPINT2* occurred in glioblastoma stem cells.[15] As MET signaling is required for the maintenance of cancer stem cells,[9,28,29] insufficiency of HAI-2/SPINT2 might contribute to the maintenance of cancer stem cells in glioblastoma through dysregulated proteolytic activation of proHGF. Further studies to explore the proHGF-activating mechanism in glioblastoma stem cells will be required.

Epigenetic silencing of the *SPINT2* gene by hypermethylation has been reported in several malignancies, including hepatocellular carcinoma, renal cell carcinoma, medulloblastoma, melanoma, esophageal carcinoma, and gastric carcinoma.[16,21,30–34] Patients suffering from esophageal cancer with *SPINT2* hypermethylation had shorter survival.[33] In this study, we also undertook MSP analyses of a series of resected human glioma tissues. The analyses confirmed the high incidence of *SPINT2* methylation in high-grade gliomas (grades III and IV). However, we did not observe a correlation between the methylation status and patients' overall survival in the current analysis. This might be due to the limited number of cases analyzed, and we could not undertake detailed stage-controlled or genetic signature-controlled subgroup analysis of the glioma cases in this study. Further analysis of the clinical relevance of the *SPINT2* methylation status will require a larger cohort of glioma cases in a future study. As *SPINT2* silencing might confer resistance to therapies through activation of HGF/MET signaling in glioma cells,[9] analysis of the impact of *SPINT2* methylation status on resistance to radiation or drugs such as temozolomide will also be required. Alternatively, the expression level of the target proteases of HAI-2/SPINT2, such as HGFA, in glioma cells with *SPINT2* methylation could be an important determinant in the patient's prognosis. In fact, our analysis of TCGA database suggested that higher *HGFAC* expression level predicted shorter survival of patients with high-grade glioma.

In summary, the *SPINT2* gene is highly methylated in high-grade glioma. Forced overexpression of HAI-2/SPINT2 protein in glioblastoma cells suppressed the growth in vitro and downregulated MET phosphorylation both in vitro and in vivo. Further studies of the roles of HAI-2/SPINT2 protein in gliomas and identification of the protease regulated by HAI-2/SPINT2 in glioma cells could supply novel molecular targets for glioma treatment.

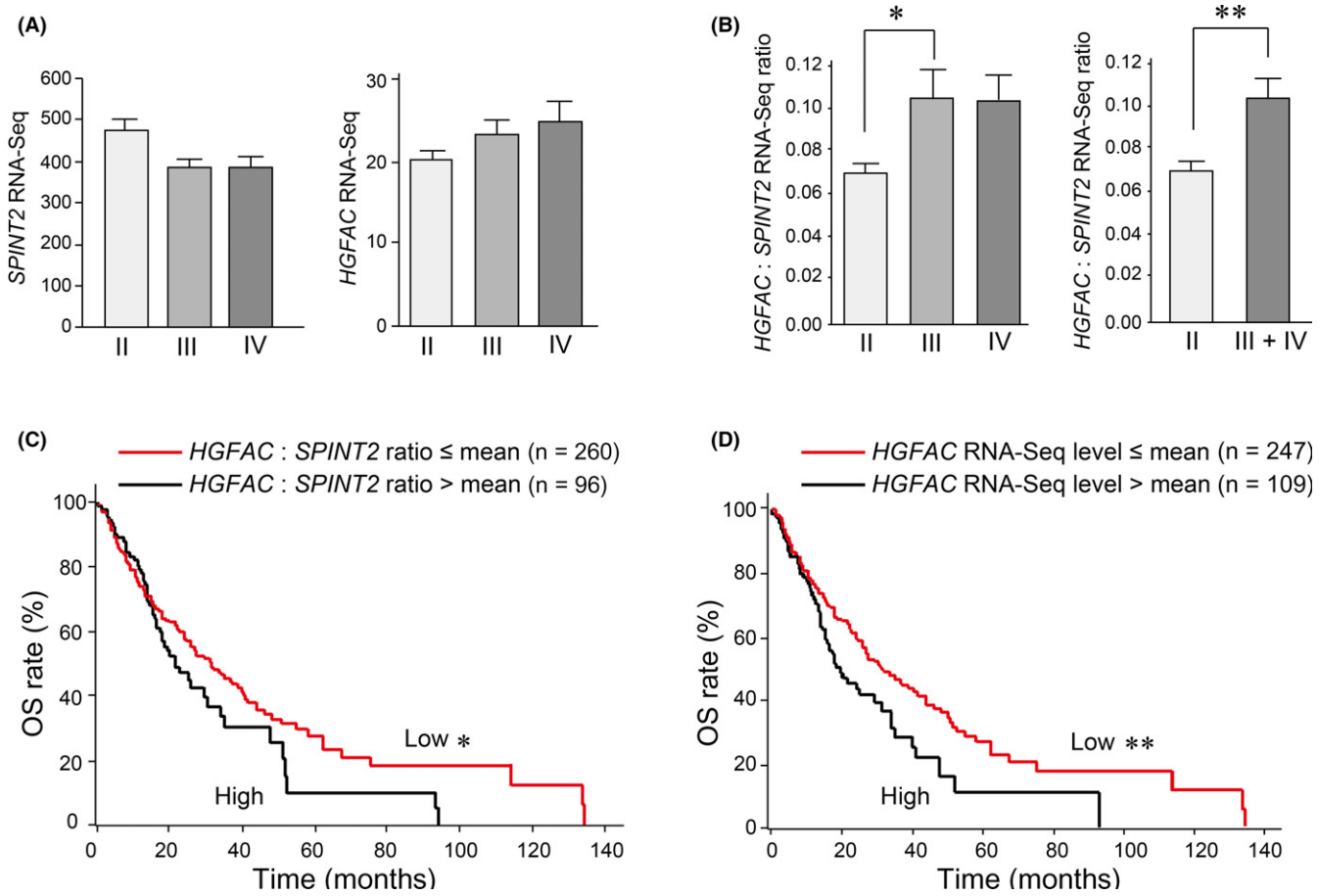

FIGURE 5 *HGFAC*:*SPINT2* expression ratio and its relationship to the survival of high-grade glioma patients. A, Expression of *SPINT2* and *HGFAC* in grade II (n = 320), grade III (n = 206), and grade IV (n = 160) gliomas. The expression levels (RNA sequencing data) of each gene were retrieved from The Cancer Genome Atlas. Differences between groups were not statistically significant in either graph. B, *HGFAC*/*SPINT2* expression ratio in low-grade (II) and high-grade (III + IV) gliomas. **P* = .027; ***P* = .039 (Mann-Whitney *U* test). Error bar indicates SEM. C, Kaplan-Meier analysis of the *HGFAC*:*SPINT2* expression ratio (left panel) or *HGFAC* RNA sequencing data (right panel) and outcomes from 356 high-grade glioma cases. **P* = .105; ***P* = .010 (log-rank test). High expression of *HGFAC* (ie, >mean) was associated with decreased overall survival (OS)

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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