Abstract. SE translocation (SET) oncoprotein, an inhibitor of protein phosphatase 2A, is abnormally expressed in many cancers. In this study, SET was aberrantly upregulated in gastric cancer (GC) compared with control tissues. Clinicopathological analysis showed that SET expression was significantly correlated with pathological grade (p=0.002), lymph node stage (p=0.014), and invasive depth (p=0.022). Kaplan-Meier analysis indicated that patients with high SET expression showed poorer overall survival rates than those with low SET expression. Moreover, SET knockdown downregulated GC cell proliferation, colony formation, tumorigenesis, and metastasis. The biological effect of SET on proliferation and invasion was mediated by inhibition of protein phosphatase 2, which in turn, activated Akt. Taken together, our results suggested that SET overexpression is associated with GC progression, and it might be a potential diagnostic marker for GC, thereby a possible target for GC drug development. 

Introduction

Gastric cancer (GC) is the fourth most common malignancy and third leading cause of cancer-related death worldwide, thereby representing a global cancer burden (1,2). Although its incidence is decreased, the outcomes for GC patients remain unsatisfactory due to lack of effective biomarkers to detect early GC and predict both chemosensitivity and relapse. During the early disease, GC patients experience non-specific symptoms, and most patients are diagnosed with advanced GC because of lacking early-stage symptoms; late-stage diagnoses are generally considerably late for effective treatment and result in 5-year survival rate of <20% (3). Therefore, new therapies toward molecular targets should be developed to improve the clinical outcome in GC.

Protein phosphatase 2A (PP2A), one of the main serine-threonine phosphatases, negatively regulates numerous signal transduction pathways and functions as a tumor suppressor in several cancers (4). Consistent with its role as a tumor suppressor, PP2A is crucial in the regulation of survival, cell cycle progression, and differentiation by negatively regulating the PI3K/Akt pathway (5), and dephosphorylating and inactivating ERK and MEK1 family kinases. Aberrant expression, mutations, and somatic alterations of PP2A scaffold and regulatory subunits are frequently found in human breast, lung, colon, and skin cancers (6). Thus, reactivation of PP2A activity based on its tumor suppressor properties is considered an attractive therapeutic strategy for human cancer treatment (7,8).

SE translocation (SET) oncoprotein, an endogenous inhibitor of PP2A (9), was initially identified as a component of SET-CAN fusion gene produced by somatic translocation in acute, undifferentiated leukemia (10). It is a multifunctional protein, which belongs to the NAP1 family of histone chaperones. SET binds to nucleosomal histones and inhibits histone acetylation by masking histone tails as component of the INHAT complex (11). Recent reports revealed aberrant upregulation of SET in multiple cancer types; this protein is also a potential therapeutic target for cancer (12-18). SET is also involved in cell proliferation, apoptosis, and invasive behavior because it controls histone acetylation, β-adrenergic receptor phosphorylation, and granzyme activity (11-18). However, the role of SET in GC tumorigenesis and metastasis remains elusive.

In this study, we analyzed the expression of SET in GC specimens and established GC cell lines by western blot...
analysis and immunohistochemistry. We manipulated SET levels in different types of GC cells and measured their effect on tumorigenesis and metastasis in vitro and in vivo. To address possible mechanisms, the effect of SET on metastasis morphology and function was investigated. On the basis of our results, we propose that SET is essential in tumorigenesis and metastasis of GC by inhibiting PP2A.

Materials and methods

Patients. Two independent GC cohorts in tissue microarray (TMA) were utilized in this study. The training cohort TMA was purchased from Wuhan Iwill Biological Technology Co., Ltd. (Wuhan, China). It included 102 tissues of patients, and 52 paired non-cancerous normal tissues from these patients were also obtained. The array dot diameter was 1.5 mm, and each dot represented a tissue spot from one individual specimen that was selected and pathologically confirmed. Six pairs of tumor and adjacent normal gastric tissues were collected immediately after surgical resection and stored in liquid nitrogen until further use. For western blot assay, tissue specimens were ground in liquid nitrogen-cooled mortar, tissue powder was suspended in lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, complete protease inhibitor cocktail] and cleared by centrifugation. The samples were used by the approval of the Institutional Review Board of Hubei University of Medicine and Dongfeng General Hospital Affiliated to Hubei University of Medicine. All tissue samples were obtained with written informed consent from patients at the Dongfeng General Hospital.

Cell culture. The GC cell lines MKN74, SGC7901, BGC823, and MGC803 and normal human gastric epithelial cell lineGES-1 were purchased from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). SGC7901, BGC823, and GES-1 were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA). GES-1 were purchased from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured in RPMI-1640 (Hyclone Laboratories, Inc., Logan, UT, USA), 100 U/ml penicillin, 100 mg/ml streptomycin. MKN74 and MGC803 cells were cultured in RPMI-1640 (Hyclone Laboratories, Inc.) supplemented with 10% FBS, 100 U/ml penicillin (Amresco, Cleveland, OH, USA), streptomycin (100 mg/ml) (Amresco). All cells were incubated in a humidified atmosphere with CO₂ at 37°C.

Real-time quantitative PCR (qPCR). Expression of the SET gene was examined by real-time polymerase chain reaction (PCR) normalized to expression of β-actin. Total RNA was extracted from cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. qPCR analysis of SET was performed with 2 µg of total RNA and ReverTra Ace qPCR RT kit (Toyobo Co., Ltd. Life Science Department, Osaka Japan). Mixed 2 µg RNA, 4 µl 5X RT buffer, 1 µl RT enzyme mix, 1 µl primer mix, and nuclease-free water ≤20 µl volume. The reverse transcription step as follows: 37°C for 15 min, 98°C for 5 min, then stored at -20°C. For qPCR, we used SET gene forward primer, 5'-AAATATAACAAACTCCGCCCTTCATCACC-3'; reverse primer, 5'-CAGTGCTCCTCTCATCTTCTC-3'. The sequences were as follows: NC short hairpin RNA, 5'GTTTCTCCACGTTGCAGGTT-3'; shSET 1#, 5'-GCCCTTCCGTTCTGACAAATAT-3';
shSET 2#, 5'-GTGTACACAAAGGATTTGTGT-3'; and shSET 3#, 5'-GGTGATCCATCTCGAAGTCC-3'. Also, 48 h after transfection the cells were harvested for western blot analysis, cell viability, and MTT assay. Cells were transfected with the lentivirus system (GenePharma). Transfection efficiency was assessed by western blot analysis and cell sorting, which was also used to select stably transfected cells.

Cytotoxic assay and cell viability. Cells were seeded into a 96-well plate and pre-cultured for 24 h, and then transfected with plasmid or siRNA for 24 or 48 h. Cell cytotoxicity was determined by MTT assay. The absorbance was measured at 570 nm by automated microplated reader (Bio-Tek, VT, USA), and the cell death rate was calculated as followed: inhibition rate (%) = (average A570 of the control group - average A570 of the experimental group) / (average A570 of the control group - average A570 of the blank group) x 100%. Cell viability was estimated by trypan blue dye exclusion (20).

Soft-agar colony formation assay. Cells were suspended in 1 ml of RPMI-1640 or DMEM containing 0.3% low-melting-point agarose (Amresco) and 10% FBS, and plated on a bottom layer containing 0.6% agarose and 10% FBS in 6-well plate in triplicate. After 2 weeks, plates were stained with 0.2% gentian violet and the colonies were counted under light microscope (IX70; Olympus Corp., Tokyo, Japan) (20).

Murine models. Equal number of female and male nude immunodeficient mice (nu/nu), 6-8 weeks old, were purchased from Hunan SJA Laboratory Animal Co., Ltd., and maintained and monitored in a specific pathogen-free environment. All animal studies were conducted according to protocols approved by the Hubei University of Medicine Animal Care and Use Committee, complying with the rules of Regulations for the Administration of Affairs Concerning Experimental Animals (Approved by the State Council of China). The mice were injected subcutaneously with SGC7901 (5x10⁶ cells/mice) stably transfected with shSET or NC vectors in 100 µl PBS. Injections were subcutaneously into the right flank of each mouse. Tumors were measured with a caliper and the volume calculated as follows: 4π/3 x (width/2)² x (length/2), representing the 3-dimensional volume of an ellipse. After 30 days, the mice were euthanized by cervical dislocation and tumor tissues were excised, imaged, and weighed.

Invasion assay. An invasion assay was carried out using 24-well plate (Corning, Inc., Corning, NY, USA). A polycarbonate filter (8-µm pore size) (Corning) was coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The lower chamber was filled with medium containing 20% FBS as chemoattractant. The coated filter and upper chamber were placed over the lower chamber. Cells (1x10⁷ cells/well) were seeded onto the upper chamber wells. After incubation for 20 h at 37°C, the filter was fixed and stained with 2% ethanol containing 0.2% crystal violet (15 min). After being dried, the stained cells were enumerated under light microscope at 10x objective. For quantification, the invaded stained cells on the other side of the membrane were extracted with 33% acetic acid. The absorbance of the eluted stain was determined at 570 nm.

Wound healing assay. Cells (4x10⁵ cells/ml) were seeded in a 6-well plate and incubated at 37°C until 90-100% confluence. After the confluent cells were washed with a 200-µl pipette tip, followed by washing with PBS, and then treated with serum-free medium. After 24 h of incubation, the cells were fixed and stained with 2% ethanol containing 0.2% crystal violet powder (15 min), and randomly chosen fields were photographed under a light microscope at 4x objective. The number of cells migrated into the scratched area was calculated.

PP2A activity assay. PP2A immunoprecipitation phosphatase assay kit (Upstate, Temecula, CA, USA) was used to measure phosphate release as an index of phosphatase activity according to the manufacturer's instructions. Briefly, 100 µg protein isolated from cells was incubated with 4 µg anti-PP2A monoclonal antibody overnight. Protein A agarose beads (40 µl) were added and the mixture was incubated at 4°C for 2 h. Subsequently, the beads were collected and washed three times with 700 µl of ice-cold TBS and one time with 500 µl Ser/Thr assay buffer. The beads were further incubated with 750 mM phosphopeptide in assay buffer for 10 min at 30°C with constant agitation. Of Malachite Green Phosphate Detection solution (100 µl) was added and the absorbance at 650 nm was measured on a microplate reader (21).

Statistical analysis. All experiments were repeated at least three times and the data are presented as the mean ± SD unless noted otherwise. Differences between data groups were evaluated for significance using Student's t-test of unpaired data or one way analysis of variance and Bonferroni post hoc test. P-values <0.05 indicate statistical significance.

Results

SET is overexpressed and associated with poor prognosis in GC. We first investigated SET prevalence in a panel of 4 human GC cell lines (MKN74, SGC7901, BGC823 and MGC803) and 1 normal human gastric epithelial cell line GES-1. Real-time quantitative PCR (qPCR, Fig. 1A) and western blot analysis (Fig. 1B) showed high level of SET in 46% of tumor samples (47 of 102), and the adjacent normal tissues exhibited low (or moderate) SET staining (21 of 25) (Fig. 1F and G). These results suggested that SET
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Survival analysis revealed that GC patients with high SET expression exhibited poorer overall survival than those with low SET expression (p=0.037; Fig. 1H). Furthermore, we analyzed the relationship between SET expression levels and clinicopathological characteristics. As shown in Table I, SET expression and sex, age, clinical stage, or tumor size at diagnosis showed no statistically significant correlations (p>0.05). Nevertheless, statistically significant correlations among high levels of SET expression were found with pathological grade (p=0.002), lymph node stage (p=0.014), and invasive depth (p=0.022). Altogether, these data suggested that SET is overexpressed in GC, and high level of SET expression is a prognostic predictor of progression and poor prognosis of GC patients.

Knockdown of SET inhibits GC cell proliferation and tumorigenesis. We also evaluated the functional role of SET in GC proliferation and tumorigenesis. GC cell lines were transfected with negative control (NC) or SET-specific shRNA (shSET) vectors (Fig. 2A). MTT and clonogenic assay were used to investigate the involvement of SET in tumorigenesis. Knockdown of SET significantly decreased
cell viability (Fig. 2B) in MGC803, SGC7901, and BGC823 cells compared with that of the NC group. Furthermore, colony formation was reduced after transfection with SET shRNA (Fig. 2C). To further determine the importance of SET in tumorigenesis, we investigated the effect of SET knockdown on tumor growth in vivo. SGC7901 cells stably expressing NC or shSET were injected subcutaneously into the right flanks of nude mice. SET knockdown substantially inhibited in vivo tumor growth on SGC7901 cells (fig. 2D-f).

Knockdown of SET suppresses GC cell invasion and migration. To examine the role of SET in GC cell metastasis, we evaluated the effect of SET knockdown on GC cell invasion and migration by Transwell and wound healing assays. As shown in Fig. 3A, SET knockdown drastically reduced the invasive ability of MGC803, BGC823, and SGC7901 cells compared with that of NC. Wound healing assay also showed that knockdown of SET significantly decreased the migratory rate of MGC803, BGC823, and SGC7901 cells (Fig. 3B). We also evaluated the effects of SET on the expression of several proliferative and invasive protein markers. Western blot analysis revealed that knockdown of SET markedly suppressed the protein expression of c-Myc and cyclin D1 and decreased the expression of matrix metalloproteinase 2 (MMP2) (Fig. 3C). c-Myc is a target of PP2A (23). Moreover, SET participates in the regulation of cellular molecular processes by inhibiting the tumor suppressor PP2A (9). Thus, we analyzed whether SET silence can alter the effects of PP2A in GC cells. SET depletion significantly upregulated the phosphatase activity of MGC803, BGC823 and SGC7901 cells (Fig. 3D). In addition, we used western blot analysis to investigate Akt phosphorylation (pAkt) and found that SET silence also reduced pAkt, a critical PP2A target (Fig. 3C).

Inhibition of PP2A is essential for SET-induced proliferation and metastasis. To further evaluate whether SET-induced cell proliferation is caused by the inhibition of PP2A activity, we compared cell proliferation in shSET-transfected GC cells and NC-transfected GC cells in the presence and absence of PP2A inhibitor, that is, okadaic acid (OA). OA significantly reversed the cell proliferation inhibited by SET silence (Fig. 4A). In addition, we compared cell proliferation in shSET-transfected GC cells and NC-transfected GC cells in the presence and absence of PP2A activator FTY720. FTY720 significantly enhances

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\(^a\) Chi-square test.
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the cell proliferation inhibited by SET silence (Fig. 4B).
Furthermore, OA also significantly reversed the cell invasion and migration inhibited by SET silence (Fig. 4C and D). Hence, inhibition of PP2A is, at least in part, essential for SET-induced proliferation and metastasis.

Discussion

As one of the most common human malignancies in China, GC remains as a challenging disease. Complete resection is the primary treatment of localized tumors, but patients who are newly diagnosed with GC tend to present advanced and often incurable diseases (24). Therefore, novel diagnostic markers, molecular pathways implicated in GC pathogenesis, and targeted therapy should be developed urgently. This report is the first to demonstrate that SET expression is significantly upregulated in GC cell lines and cancer tissues at both protein and mRNA levels in comparison with those of normal cells and paired adjacent non-cancerous gastric tissues. We found that high expression of SET was correlated with pathological grade, lymph node stage, and invasive depth. SET also played an oncogenic role of promoting cell proliferation, colony formation, and metastasis. Furthermore, SET was required for tumorigenesis of GC.

SET is a predictive marker for prognosis in Wilms tumor (12), pancreatic tumor (13), prostate cancer (14), ovarian cancer (15), lung tumor (16), acute myelogenous leukemia (17), and chronic lymphocytic leukemia (18). Overexpression of SET oncoprotein in these cancer cells decreases PP2A activity and promotes cell proliferation, survival, drug resistance, invasion, and metastasis. In this study, SET was overexpressed in all GC cell lines, but showed low expression in normal gastric epithelial cells, thereby suggesting that SET has a positive role in GC formation (Fig. 1A and B). In addition, GC samples expressed high levels of SET compared with those of adjacent normal gastric tissues (Fig. 1C-F). Multivariate analysis indi-
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cated that SET was closely associated with tumor progression and poor prognosis (Fig. 1G and Table I), and SET may represent an independent prognostic biomarker for GC patients. SET knockdown attenuated the tumorigenicity of SGC7901 cells in nude mice, which suggested that SET is required in GC tumorigenesis and progression, which is consistent with previous findings (Fig. 2D-F).

Invasion and migration are important characteristics of cancer cell metastasis (25). To determine the potential mechanisms of GC, we further tested the effect of SET knockdown on migration and invasion. Wound healing and Transwell assay data indicated that silencing of SET in GC cells could efficiently inhibit the invasive capability of GC cells (Fig. 3A and B). Downregulation of SET also inhibited the expression of cyclin D1, MMP2, c-Myc, and pAkt and upregulated the activity of PP2A (Fig. 3C and D). These abnormally expressed downstream molecules of CIP2A are critical for GC proliferation and metastasis.

PP2A, one of the four major classes (PP1, PP2A, PP2B, and PP2C) of eukaryotic serine/threonine phosphoprotein phosphatases, is a key tumor suppressor that regulates survival, cell cycle progression, and differentiation-related signaling pathways with...
high relevance in human cancer (4,23). SET oncoprotein is an important binding partner of PP2A with an inhibitory function, and collected evidence supports the idea that SET-promoted tumor progression is mediated by PP2A inhibition (16). We treated OA, the inhibitor of PP2A in SET-depleted GC cells. The results showed that loss of PP2A function can reverse the inhibition effect of SET depletion in GC cell proliferation, migration, and invasion (Fig. 4A-C). These results confirmed that PP2A inactivation plays a critical role in SET-promoted GC proliferation and invasion progression. PP2A-activating drugs represent promising anticancer molecules that can be used in cancer treatment (16,26,27). Thus, targeting SET protein is a potential strategy to treat cancer by reactivation of PP2A.

In conclusion, high expression of SET is a recurrent event in GC, where it serves as a marker of reduced overall survival and a poor prognostic factor, as previously reported in other tumors. Moreover, SET depletion upregulates PP2A activity, thereby reducing cell proliferation, invasion, and migration. Our results confirmed that SET behaves as an oncoprotein in GC and could represent a novel therapeutic target in GC.
Acknowledgements

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