Efficacy of an anti-cancer strategy targeting SET in canine osteosarcoma

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ABSTRACT

Osteosarcoma (OSA) is the most common bone tumor in dogs. Protein phosphatase 2A (PP2A), an evolutionary conserved serine/threonine protein phosphatase, is a crucial tumor suppressor. SET is a PP2A inhibitory protein that directly interacts with PP2A and suppresses its phosphatase activity. SET has been reported as a contributor of wide range of human and dog tumor malignancies. However, the role of SET in canine OSA (cOSA) remains unknown. In this study, we investigated the role of SET in cOSA by using 2 cOSA cell lines: POS (primary origin) and HM-POS (metastatic origin). Knockdown (KD) of SET expression was noted to slightly suppress POS cell proliferation only. Furthermore, SET KD effectively suppressed colony formation ability of both POS and HM-POS cells. SET KD was observed to repress ERK1/2, mTOR, E2F1, and NF-κB signaling in HM-POS cells, whereas it inhibited only ERK1/2 signaling in POS. Further, it was observed that SET-targeting drug, FTY720, exerted anti-cancer effects in both POS and HM-POS cells. Moreover, the drug also enhanced the anti-cancer effect of cisplatin. The data suggested that a combination therapy, based on SET targeting drugs and cisplatin, could be a potent strategy for cOSA.

KEY WORDS: canine, osteosarcoma, Protein Phosphatase 2A (PP2A), SET
INTRODUCTION

Osteosarcoma (OSA) is a predominant bone tumor diagnosis in dogs. Although, any dog could potentially develop OSA, but dog breeds with body weight above 40 kg are at an increased risk of developing OSA [16, 23]. Surgery, limb amputation and limb salvage/sparing, constitutes the first choice of treatment, however, more than 90% of the patients possess micro-metastasis [16, 23]. Therefore, adjuvant and non-adjuvant chemotherapy is important to improve the survival rate. In canine OSA (cOSA), adjuvant chemotherapy with cisplatin, carboplatin, or doxorubicin have been associated with increased survival rates over amputation alone [1, 20]. OSA is a rare cancer in humans, whereas its incidence rate in dogs is 27-times higher [22], suggesting cOSA as a good model for human disease. The standard adjuvant chemotherapeutic approach in human OSA comprises of a combination of cisplatin, methotrexate, and doxorubicin, and has remained relatively unaltered for years [25, 26]. Prognosis in both the species is poor and the survival rate has not improved in decades. Therefore, establishment of novel anti-cancer drugs is urgently required.

Protein phosphatase 2A (PP2A) is an evolutionally conserved serine/threonine phosphatase that suppresses the activity of multiple intracellular signals such as Akt, ERK1/2, and mTOR signaling which regulates cancer cell growth, stemness, and survival [12, 18, 19]. PP2A is reported to negatively regulate the activity of transcription factors such as c-Myc, E2F1, and NF-κB, that mediates tumor cell stemness and the cell cycle [9, 12, 19]. Inhibition of PP2A activity is required for tumor transformation and increased expression of endogenous PP2A inhibitory proteins (such as SET, CIP2A, and PME-1) contributes to lower PP2A activity in cancer cells [5, 21]. We have previously reported that SET contributes to tumor malignancy in dogs
including lymphoma, melanoma, and mammary tumor [8, 10, 12]. In these cancers, SET-targeting drugs, such as FTY720 and OP449; dissociates SET from PP2A and exhibits anti-cancer effects via PP2A reactivation. Therefore, SET is considered to be an attractive target for anti-cancer drug.

Here, we demonstrated that SET contributes to an increase in phosphorylation/activity of ERK1/2, mTOR, E2F1, c-Myc, and NF-κB in cOSA cell lines. Moreover, FTY720 displayed anti-cancer effects and enhanced cisplatin’s effect in cOSA cell lines. The results suggested the potential clinical application of SET inhibitors for cOSA.

**MATERIALS AND METHODS**

**Cell Culture:** Canine osteosarcoma cell lines, POS and HM-POS [2, 11] were cultured in RPMI 1640 containing 10% FBS and 1x antibiotic/antimycotic (Life Technologies, Carlsbad, CA, USA).

**Antibodies:** Antibodies were obtained from the indicated supplier: anti-SET (sc-133138), anti-p70S6K (sc-230), anti-E2F1 (sc-193), anti-NF-κB p65 (sc-372), anti-c-Myc (sc-764) (Santa Cruz Biotech, Santa Cruz, CA, USA), anti-phospho-ERK p42/p44 (9101), anti-ERK p42/p44 (9107), anti-MEK1/2 (4694), anti-phospho-MEK1/2 (9154), anti-phospho-Thr389 p70S6K (9234), anti-phospho-Ser473 Akt1 (4060), anti-phospho-Thr308 Akt1 (13038), anti-Akt (2920), anti-phospho-Ser536 NF-κB p65 (3033) (Cell Signaling, Danvers, MA, USA), anti-PP2Ac (07-324) (Merck Millipore, Burlington, MA, USA), anti-p97/VCP
shRNA and Lentivirus Production: shRNA sequences and procedure for lentivirus production was previously described [8]. Briefly, to produce lentiviruses, 3 µg of pLVSIN, 2.3 µg of a packaging plasmid (psPAX2) and 1.3 µg of a coat-protein plasmid expressing vesicular stomatitis virus G protein (pMD2.G) were transfected into Lenti-X 293T cells (Takara Bio, Shiga, Japan) cultured in 60-mm dishes using PEI Max (Polysciences, Warrington, PA, USA) according to the manufacturer’s instruction. Viral supernatants were collected after 48 hr, and after filtering (0.22 µm), were added to cells for 12 hr.

Immunoblotting: 1.0 × 10^6 cells were cultured in RPMI 1640 containing 10% FBS and 1x antibiotic/antimycotic on 6 cm dish, and proteins were extracted after 24 hours. Immunoblotting was performed as previously described [8]. Briefly, cells were lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 5 mM EGTA, 1% Triton X100, 1 mM Na₃VO₄, 20 mM sodium pyrophosphate and Roche’s complete protease inhibitor cocktail. The proteins were separated by SDS-PAGE and transferred onto nitrocellulose membrane (Wako, Osaka, Japan). Membranes were blocked with 0.5% skim milk and treated with primary antibodies. Immunoreactive bands were detected using Western Lightning ECL Pro (PerkinElmer, Freiburg, Germany) and visualized using a LAS-3000 luminescent image analyzer (Fujifilm, Tokyo, Japan).
Cell Proliferation and Cell Viability Assay: 1.0 × 10^4 cells were seeded on 24-well plates and were cultured in RPMI 1640 containing 10% FBS and 1x antibiotic/antimycotic for 4 days. Cell Counting Kit-8 (CCK8, Dojindo, Kumamoto, Japan) was used to analyze cell proliferation according to the manufacturer’s instruction. For cell viability analysis, 1.0 × 10^4 cells were seeded on 24-well plate, and drugs were added to the medium after 24 hr. After additional 72 hr, CCK8 was used to analyze cell viability.

Colony Formation Assay: 1.0 × 10^2 cells were seeded on 6-well plates and were cultured in RPMI 1640 containing 10% FBS and 1x antibiotic/antimycotic. After 10 days, cells were fixed with 99.5% ethanol, colonies were stained with Giemsa solution, and the number of colonies was counted.

Statistical Analysis: Statistical analysis was performed using SigmaPlot (HULINKS). The results are expressed as means ± S.E. Student’s t test was used for comparison between two groups. Groups more than three were compared using one-way analysis of variance, after which Fisher LSD test was used. For all analyses, a probability value of P < 0.05 was considered statistically significant.

RESULTS

To analyze the role of SET in cOSA, 2 cOSA cell lines, POS (primary origin) and HM-POS (established from nude mice lung metastatic site of POS cells) were used [2, 11]. The protein levels of SET and PP2A were not significantly different between the POS and HM-POS cells (Fig. 1A and B). For suppressing SET expression,
non-target shRNA (shNT) and SET-targeting shRNA (shSET) were stably expressed in
the cOSA cell lines. SET protein expressions were observed as effectively suppressed
by shSET as compared to shNT (Fig. 1C and D). Furthermore, the effect of SET
knockdown (KD) on tumorigenic phenotype of cOSA cell lines was analyzed. SET KD
was noted to slightly suppress POS’s cell proliferation only (Fig. 2A). On the other
hand, SET KD effectively suppressed the colony formation ability of both POS and
HM-POS cells (Fig. 2B and C). The data suggested that SET plays an important role in
tumor malignancy of cOSA.

Further, the effects of SET KD on cell signaling were analyzed. SET KD has
been reported to suppress multiple cellular signaling including Akt, ERK1/2, and
mTOR and also the activity of transcriptional factors like E2F1, NF-κB, and c-Myc in
PP2A dependent manner [9, 12, 14, 19]. We observed that SET KD suppressed ERK1/2
phosphorylation in both POS and HM-POS cells, without altering the activity of an
upstream kinase, MEK1/2 (Fig. 3A-D). The data indicated that SET inhibits PP2A
complex, which is responsible for ERK1/2 dephosphorylation. A decreased in p70S6K
(mTOR complex 1 (mTORC1) signaling activity marker) phosphorylation in HM-POS
cells was also observed (Fig. 3E-F). PDK1 and mTORC2 mediate the phosphorylation
of Akt’s Thr308 and Ser473, respectively [15]. SET KD, however, did not affect Akt
phosphorylation in both the cell lines (Fig. 3G-H).

Furthermore, we analyzed the effect of SET KD on the phosphorylation levels
of transcriptional factors E2F1, NF-κB, and c-Myc (Fig. 4A-F). In POS cells, the
phosphorylation and protein levels of these transcriptional factors were not affected.
Whereas, SET KD suppressed E2F1 and NF-κB p65 phosphorylation in HM-POS cells
and the sites were associated with protein stability and activity, respectively.
Interestingly, SET KD increased c-Myc protein level in HM-POS cells. The results suggested that enhanced activity of ERK1/2, mTORC1, E2F1, and NF-κB signaling might be associated with SET-mediated cOSA malignancy.

Although SET KD decreased ERK1/2 phosphorylation levels in both POS and HM-POS cells, it suppressed cell proliferation only in POS cells. To clarify this discrepancy, we analyzed the effects of ERK1/2 inhibitor, FR180204, on cell proliferation and colony formation ability. FR180204 suppressed cell proliferation and colony formation in both cell lines, and POS cells showed significantly higher sensitivity for this drug (Fig. 5A-C). These data indicate that tumorigenic potential of POS cells strongly depend on ERK1/2 activation.

FTY720 (Fingolimod), a sphingosine analog, is used as an immunosuppressant in human multiple sclerosis patients. FTY720 has been reported to directly interact with SET and restore PP2A activity [17]. We have previously reported that FTY720 selectively exerts anti-cancer effects on SET-KD-sensitive canine tumor cell lines [12] The anti-cancer effect of FTY720 on cOSA (Fig. 6A) was analyzed to reveal that it affects both POS and HM-POS cells in a dose-dependent manner. The anti-cancer effect of FTY720 was observed as almost same between 2 cell lines. Cisplatin is one of the most commonly used drugs for cOSA treatment. We analyzed if its combination with FTY720 exerts any additional anti-cancer effects on cOSA cells (Fig. 6B). Co-treatment with FTY720 and cisplatin exhibited effectively suppressed cOSA cell survival rate than FTY720 or cisplatin treatment alone. The result suggested that the combination therapy, based on SET targeting drugs and cisplatin, could act as a potent strategy for cOSA.
DISCUSSION

In this study, SET was suggested as a novel anti-cancer target for cOSA. Consistent with our previous report in human cancer cell lines [9], SET KD affects on colony formation ability, a marker for cancer cell stemness, than cell proliferation. It has been reported previously that SET expression level is related to PP2A inhibitory ability and tumor malignancy [6]. The protein expression levels of SET and PP2A are almost similar between POS and HM-POS cells. The observed effects of SET KD and SET-targeting drug on tumorigenic ability were also not much different between these 2 cell lines. However, a difference in the effect of SET KD on cell signaling was noted. SET KD was observed to suppress ERK1/2 signaling in both cell lines, whereas it also suppressed mTORC1, E2F1, and NF-κB signaling in HM-POS cell.

Abnormal activation of ERK1/2 signaling has been implicated as tumor proliferation, migration, and metastasis in human OSA [4]. Our data suggests that ERK1/2 activation plays an important role in SET-mediated cOSA tumor malignancy. ERK1/2 inhibitor suppressed cell proliferation and colony formation of both cell lines, but the sensitivity for the drug is much higher in POS cells compared with HM-POS cells, may be a reason for that only POS cells showed decreased cell proliferation by SET KD. ERK1/2 has been reported as a substrate of PP2A [24]. It was observed that MEK1/2 (an upstream kinase of ERK1/2 phosphorylation) activity was not affected by SET KD, suggesting that re-activation of PP2A was involved in decreased ERK1/2 phosphorylation by SET KD.

It remains unclear why SET KD affects multiple cellular signaling only in HM-POS, even though SET protein levels were almost same in both the cell lines. In humans, 2 highly homological SET isoforms (SETα and SETβ) exists and high
expression ratio of SET$\alpha$/SET$\beta$ is observed to be associated with poor prognosis of chronic lymphocytic leukemia patient [3]. We have previously reported in human gastric cancer cell lines that SET KD-induced suppression of tumorigenic potential was rescued by SET$\alpha$, but not by SET$\beta$ [9]. Therefore, expression ratio of SET isoforms might be an important parameter in understanding the role of SET in tumor cell. In canine, at least 4 SET isoforms are reported [27]. Canine SET$\alpha$ protein has approximately 94% homology with human SET$\alpha$. In this study, we utilized shRNA targeting all the canine SET isoforms. Therefore, difference in expression pattern of SET isoforms between the cell lines might affect the outcome of SET KD.

Drug sensitivity screening using HeLa cells revealed that among PP2A inhibitory proteins, SET depletion was most effective to result in cells sensitivity against anti-cancer drugs [13]. In this study, SET-targeting drug FTY720 was revealed to enhance the anti-cancer effects of cisplatin on cOSA cell lines. Cisplatin induces cancer cell death by preventing DNA replication and suspending cell division [7]. We have previously reported in human cell lines, that SET KD effectively suppresses cancer cell stemness over cell proliferation [9]. Consistent with this, SET KD effectively decreased colony formation ability of cOSA cells, but only slightly affected cell proliferation. Thus, SET is an effective target for combination chemotherapy for cOSA due to the difference in molecular mechanisms between SET-targeting drug and cisplatin, inhibition of stemness and cell cycle, respectively.

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**FIGURE LEGENDS**

**Figure 1.** Expression of SET protein in canine osteosarcoma cell lines

(A, B) SET and PP2Ac protein levels were analyzed by immunoblotting. Representative images (A) and quantitative data (B) from three independent experiments are shown. VCP was used as a loading control. The protein levels were normalized to POS as 100%.

(C, D) The effect of non-targeting shRNA (shNT) and SET targeting shRNA (shSET) on SET protein level was determined by immunoblotting. Representative images (C) and quantitative data (D) from three independent experiments are shown. The SET protein levels were normalized to that of shNT as 100%. *$P < 0.05$ vs. shNT.

**Figure 2.** SET knockdown exerts anti-tumor effects on canine osteosarcoma cell lines

(A) Non-target shRNA (shNT) and SET-targeting shRNA (shSET) were stably expressed in POS and HM-POS cells. Cell proliferation was analyzed by Cell Counting Kit-8 (CCK8). Quantitative data from three independent experiments with biological duplicates are shown. The cell proliferation rates are normalized to that at Day 1 of shNT as 100%. *$P < 0.05$ vs. shNT.

(B, C) Colony formation ability of POS and HM-POS cells expressing shNT and shSET was analyzed. Representative image (B) and quantitative data (C) from three independent experiments with biological duplicates are shown. *$P < 0.05$ vs. shNT.

**Figure 3.** The effect of SET knockdown on cell signaling
The effect of SET KD on the phosphorylation and protein levels of ERK1/2 (A, B), MEK1/2 (C, D), p70S6K (E, F), and Akt (G, H) was analyzed by immunoblotting. Representative image and quantitative data from three independent experiments are shown. Band densities were normalized to that of shNT as 100%. *$P < 0.05$ vs. shNT.

**Figure 4.** The effect of SET knockdown on the transcriptional factor activity

The effect of SET KD on the phosphorylation and protein levels of E2F1 (A, B), NF-κB (C, D), and c-Myc (E, F) was analyzed by immunoblotting. Representative image and quantitative data from three independent experiments are shown. Band densities were normalized to that of shNT as 100%. *$P < 0.05$ vs. shNT.

**Figure 5.** The effect of ERK1/2 inhibitor on cell proliferation and colony formation ability of canine osteosarcoma cell lines

(A) POS and HM-POS cells were cultured without (Cont) or with 100 µM of FR180204 (FR) for 72 hr, and the cell viability was analyzed by CCK8. *$P < 0.05$.

(B, C) Colony formation ability of POS and HM-POS cells treated with 100 µM of FR for 10 days was analyzed. Representative image (B) and quantitative data (C) from three independent experiments performed biological duplicate are shown. *$P < 0.05$.

**Figure 6.** FTY720 exerts anti-cancer effect on cOSA cells and enhances the effects of cisplatin

(A) POS and HM-POS cells were treated with indicated dose of FTY720 for 72 hr, and the cell viability was analyzed by CCK8.
(B) POS and HM-POS cells were treated with or without FTY720 (5 µM) and/or cisplatin (1 µM). After 72 hr, the cell viability was analyzed by CCK8. Cell viability was normalized to that without treatment as 100%. Quantitative data from two independent experiments with biological duplicates are shown. *$P < 0.05$. 
Fig. 1
Fig. 2

(A) Cell proliferation (%) for POS and HM-POS treatments. The y-axis shows cell proliferation in percentage, while the x-axis represents time in days.

(B) Images showing colony number (%). The images compare shNT and shSET treatments for both POS and HM-POS conditions.

(C) Bar graphs illustrating colony number (%) for POS and HM-POS treatments. The bars for shNT and shSET are shown, with statistical significance indicated by asterisks.
Fig. 3

(A) POS
Phospho-ERK1/2

(B) HM-POS
Phospho-ERK1/2

(C) POS
Phospho-MEK1/2

(D) HM-POS
Phospho-MEK1/2

(E) POS
Phospho-p70S6K

(F) HM-POS
Phospho-p70S6K

(G) POS
Phospho-T308 Akt

(H) HM-POS
Phospho-T308 Akt

VCP
Fig. 4

(A) POS
Phospho-E2F1  
E2F1  
VCP

(B) HM-POS
Phospho-E2F1  
E2F1  
VCP

(C) POS
Phospho-NFκB p65  
NFκB p65  
VCP

(D) HM-POS
Phospho-NFκB p65  
NFκB p65  
VCP

(E) POS
Phospho-cMyc  
cMyc  
VCP

(F) HM-POS
Phospho-cMyc  
cMyc  
VCP
Fig. 6

(A) POS

(B) POS

Cell survival rate (%) vs. FTY720 concentration for POS and HM-POS.

Comparison of cell survival rates with different concentrations of FTY720 and Cisplatin.

* Indicates statistical significance.