

Characterization of SET/I2PP2A Isoforms in Dogs

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ABSTRACT. SET is an endogenous protein phosphatase 2A (PP2A) inhibitor and is associated with a poor prognosis in human leukemia. Previously, we reported increased SET protein levels in canine lymphoma cell lines and the potential therapeutic application of SET antagonists in canine lymphoma. Here, we found that canine cells express several isoforms of the SET protein. We cloned 4 isoforms of SET, named SET α , β , γ and δ . Genomic BLAST showed that the SET genes are located on chromosomes X, 7, 1 and 8, respectively. An immunofluorescent study showed nuclear localization of SET α and β , and nuclear and cytosolic localization of SET γ and δ . We confirmed that SET α and β possess the ability to associate with PP2A. Our data reveal the existence of unique SET isoforms that should be taken into account in SET-targeting drug development studies in dogs.

KEY WORDS: cancer, canine, phosphorylation, protein phosphatase 2A, SET/I2PP2A

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The human SET gene was discovered as a component of the SET-CAN fusion gene, which was produced by a somatic translocation in a case of acute undifferentiated leukemia [17]. It has been reported that SET positively regulates multiple oncogenic pathways, and elevated SET protein levels are observed in various human tumors [4–6, 8, 12, 18, 19]. SET, also known as I2PP2A, is a potent physiological inhibitor of protein phosphatase 2A (PP2A). PP2A is a major cellular protein serine/threonine phosphatase and regulates a wide range of biological processes, including cell proliferation, apoptosis, development and motility [3]. Loss or inhibition of PP2A has revealed that it possesses a critical tumor suppressor function [2] and the SET protein directly binds with PP2A through both its N-terminus and C-terminus regions and subsequently inhibits PP2A phosphatase activity [1].

There are 2 isoforms of human SET, the 290 amino acid SET α and the 277 amino acid SET β , both of which exhibit PP2A inhibitory activity [15]. However, it is unknown whether canine cells express SET isoforms. We have previously reported that OP449, a peptide antagonist of SET, recovered PP2A activity and exerted anti-tumor effects on canine lymphoma cells [10]. In canine lymphoma cells, the effects of OP449 were dependent on SET protein levels in the cells. Therefore, it is important to identify the isoforms of canine SET and clarify whether they are functional PP2A inhibitors.

MATERIALS AND METHODS

Cell culture: Canine melanoma cell lines (CMeC1, CMeC2, KMeC and LMeC) were kindly provided by Dr. Takuya Nakagawa. The canine lymphoma cell line Ema has previously been described [10]. CMeC1, CMeC2 and Ema were grown in RPMI1640 that contained 10% fetal bovine serum (FBS) and 1 \times anti-biotic/anti-mycotic (Life Technologies, Carlsbad, CA, U.S.A.). HeLa, HEK293T and canine renal tubule epithelial cell line MDCK were grown in Dulbecco's Modified Eagle Medium (DMEM) that contained 10% FBS and 1 \times anti-biotic/anti-mycotic. PBMCs were isolated from Beagle and cultured in RPMI1640 that contained 10% FBS.

Immunoblotting: Immunoblotting was performed as previously described [13]. Briefly, cells were lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 5 mM ethylene glycol tetraacetic acid (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 PVDF membrane (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Membranes were blocked with 0.5% skim milk and treated with the following primary antibodies: anti-human SET α (Abcam #ab1183, Cambridge, UK), anti-human SET α / β (Bioss #BS-5943R, Woburn, MA, U.S.A.), anti-PP2A C subunit (Millipore #07-324, Billerica, MA, U.S.A.) and anti-VCP/p97 (GeneTex, Hsinchu, Taiwan). Bands were detected using ECL Western Blotting Detection System (GE Healthcare, Freiburg, Germany) and visualized using a LAS-3000 luminescent image analyzer (Fujifilm, Tokyo, Japan).

Cloning of canine SET isoforms and transfection: Thymus cDNA from the Beagle was used as a template for PCR. The 37-mer oligonucleotide 5'-TATTTCCG-GTGAATTCCTTTCGTGGTTTCTCACAGC-3' and the

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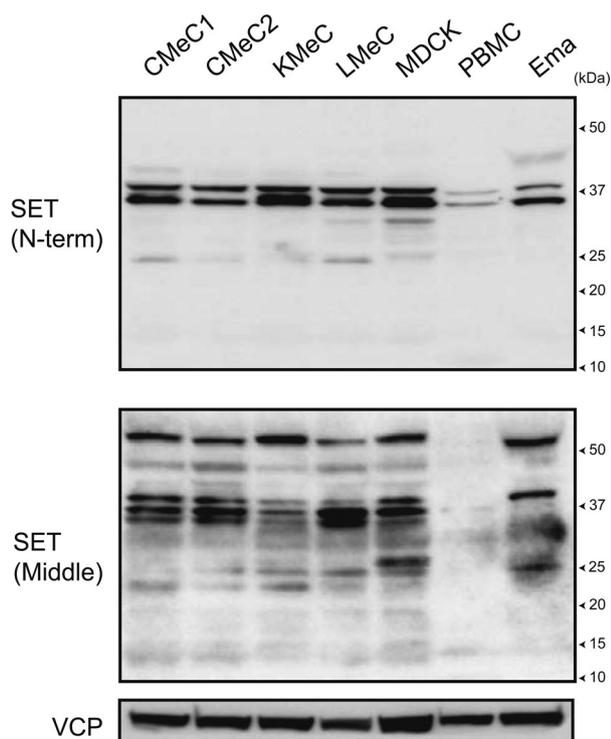


Fig. 1. SET protein expression in canine cells. SET protein expression in canine cells was analyzed by immunoblotting. Two types of anti-SET antibodies against 3–18 aa of human SET α (N-term) and 175–215 aa of human SET α (Middle) were used. VCP was used as a loading control.

36-mer oligonucleotide 5'-CTTTGTAGTCGGATCCTG-GAATCCATCAGTGTTCCTCA-3' were designed as the 5' primer and the 3' primer, respectively, to sequence canine SET variants. PfuUltra II (Life Technologies) was used for the PCR amplification, which was conducted with 40 cycles of 20 sec at 95°C, 20 sec at 60°C and 60 sec at 72°C. The PCR products were cloned into pLVSIN-EF1 α -IRES-ZsGreen Vector (Takara, Tokyo, Japan) by InFusion (Takara), according to the manufacturer's instructions. Plasmids were sequenced using BigDye Terminator v3.1 cycle sequencing kit and ABI3130 sequencer (Life technologies). Plasmids were transfected into cells using Polyethylenimine Max (Polysciences, Taipei, Taiwan), according to the manufacturer's instructions.

Immunoprecipitation: HEK293T cells transiently expressing FLAG-SET variants were lysed in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% Triton X100, 1 mM Na₃VO₄, 20 mM sodium pyrophosphate and Roche's complete protease inhibitor cocktail. The supernatants were incubated with FLAG-M2 affinity gel (Sigma, St. Louis, MO, U.S.A.). The beads were washed in a washing buffer that contained 50 mM MOPS-NaOH (pH 7.4), 150 mM NaCl, 5 mM EGTA and 1% NP-40.

Immunofluorescent staining: Cells were grown on glass coverslips and subsequently fixed with 4% paraformaldehyde

for 20 min at room temperature and blocked with 3% skim milk in phosphate buffered saline-Tween (PBS-T) (137 mM NaCl, 2.7 mM KCl, 1.76 mM KH₂PO₄, 10 mM Na₂HPO₄ and 0.05% Tween 20). After incubation with anti-SET antibodies or an anti-FLAG antibody (SIGMA) overnight at 4°C, Alexa 488 or 594-conjugated secondary antibodies (Life Technologies), Phalloidin Far-Red Fluorescent Acti-stain™ 670 (Cytoskeleton, Denver, CO, U.S.A.) and Hoechst 33342 (Dojindo, Tokyo, Japan) were added before the cells were incubated for 1 hr at room temperature. Fluorescence images were captured using a confocal laser-scanning microscope (LSM710, Zeiss, Tokyo, Japan).

RESULTS

To examine the protein expression of SET isoforms in canine cells, normal peripheral blood mononuclear cells (PBMC), melanoma cell lines CMeC1, CMeC2, KMeC and LMeC, lymphoma cell line Ema and the renal tubule epithelial cell line MDCK were lysed for immunoblotting. The major bands were observed around 37 kDa by 2 types of anti-SET antibodies against 3–18 aa of human SET α (N-term) and 175–215 aa of human SET α (Middle). The minor bands were commonly observed around 25 kDa by the 2 antibodies. Only anti-SET α (Middle) detected the major band around 50 kDa and weak bands around 15 kDa. These data indicate that canine cells express more than one SET isoform (Fig. 1).

To determine the sequence of canine SET isoforms, we cloned the protein cDNA from the Beagle thymus, as described in the Materials and Methods. Interestingly, the same primer isolated 4 types of cDNA sequences. cDNA sequences for 4 isoforms, named α , β , γ and δ , were aligned using Clustal X2 (Fig. 2). Genomic BLAST (Basic Local Alignment Search Tool) revealed that isoform α , β , γ and δ genes are 99% homologous to CanFem3.1 whole shotgun sequences NW_003726126.1, NW_003726067.1, NW_003726047.1 and NW_003726071.1 and are located on chromosomes X, 7, 1 and 8, respectively. Isoform α expresses 290 aa protein, which is the same size as human SET α . Alignment of the protein sequences of human SET α and canine SET α revealed that approximately 94% were homologous between the 2 species (Fig. 3A). In contrast, isoforms β , γ and δ have a single base insertion and express 223 aa, 112 aa and 102 aa C-terminus truncated SET proteins, respectively (Figs. 2 and 3B).

To examine whether canine SET isoforms are conformationally stable, HEK293T cells were transfected to express the canine SET isoforms, and protein levels were determined by immunoblotting (Fig. 4A). The anti-FLAG antibody detected the bands around 52 kDa for FLAGx3-SET α , around 37 kDa for FLAGx3-SET β , around 24 kDa for FLAGx3-SET γ and around 20 kDa for FLAGx3-SET δ . Although these bands are much higher than the predicted size of canine SET α , β , γ and δ , which are 33.5 kDa, 26.1 kDa, 12.6 kDa and 11.9 kDa, respectively, a similar upper shift was also observed for human SET α with FLAGx3 tag (Data not shown). Next, we examined the localization of canine SET isoforms.

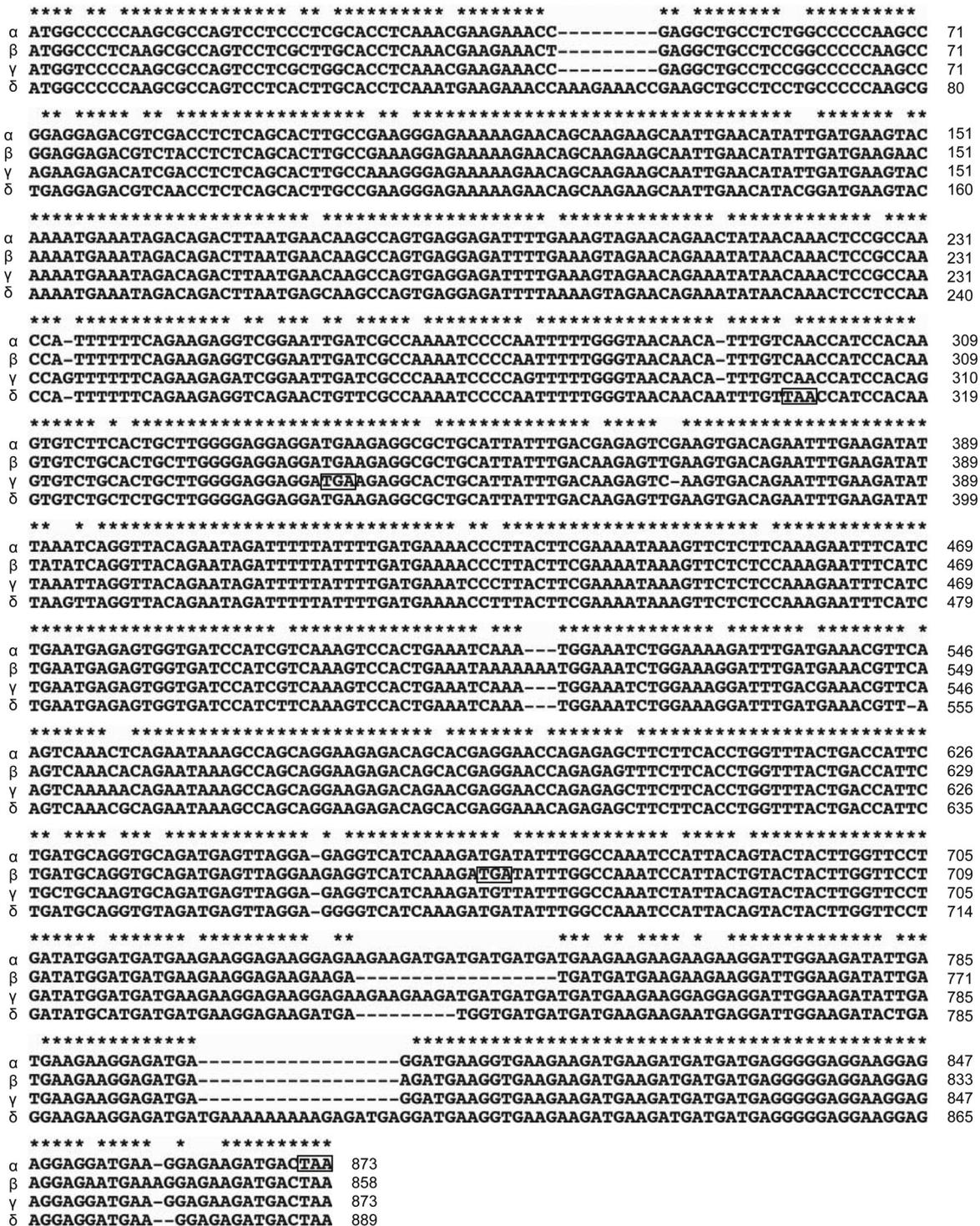


Fig. 2. Nucleotide sequence alignment of canine SET isoforms. cDNA sequences for canine SET isoforms were aligned using Clustal X2. “*”, “:” and “.” indicate “full”, “strong,” and “weak” conserved residues, respectively. The square indicates the stop codon.

Both anti-SET (N-term) and anti-SET (Middle) antibodies exhibited nuclear localization of the endogenous canine SET in CMeC1 cells (Fig. 4B). To determine the localization of

individual isoforms, HEK293T cells were transfected to express canine SET isoforms. pLVSIN-EF1 α -IRES-ZsGreen canine SET isoforms vector express both SET isoforms and

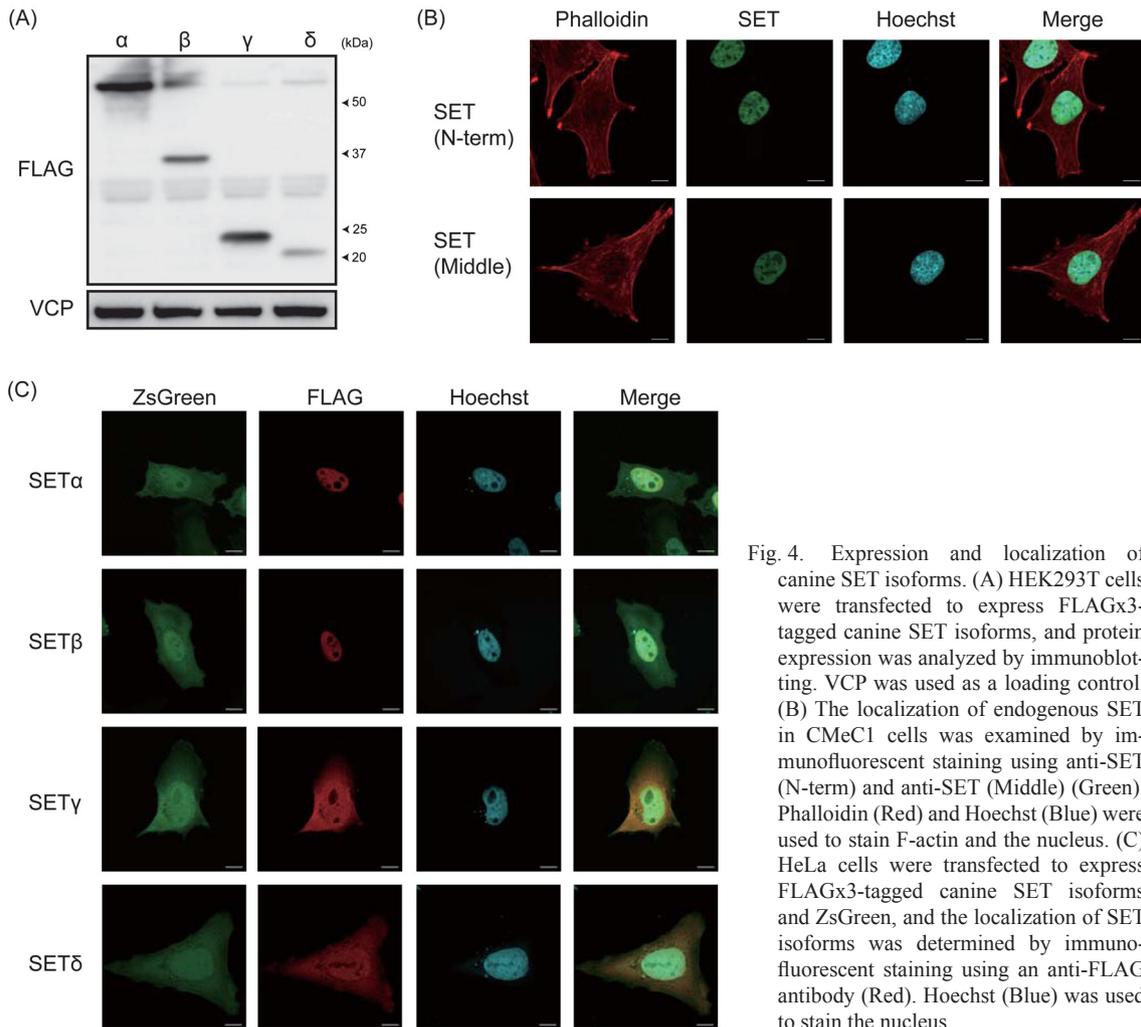


Fig. 4. Expression and localization of canine SET isoforms. (A) HEK293T cells were transfected to express FLAGx3-tagged canine SET isoforms, and protein expression was analyzed by immunoblotting. VCP was used as a loading control. (B) The localization of endogenous SET in CMeC1 cells was examined by immunofluorescent staining using anti-SET (N-term) and anti-SET (Middle) (Green). Phalloidin (Red) and Hoechst (Blue) were used to stain F-actin and the nucleus. (C) HeLa cells were transfected to express FLAGx3-tagged canine SET isoforms and ZsGreen, and the localization of SET isoforms was determined by immunofluorescent staining using an anti-FLAG antibody (Red). Hoechst (Blue) was used to stain the nucleus.

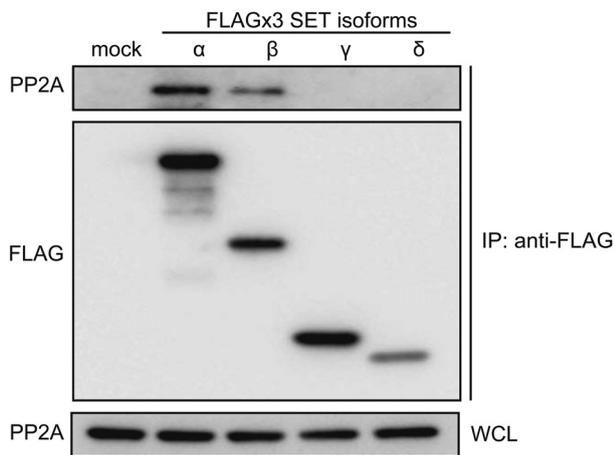


Fig. 5. Association of canine SET isoforms with PP2A. HEK293T cells were transfected to express FLAGx3-tagged canine SET isoforms, and the association with PP2A was examined by immunoprecipitation using anti-FLAG M2 beads. WCL: whole cell lysates.

whether these bands correspond to another SET isoform or non-specific binding. Further observations are necessary to clarify this point.

Human SET isoforms are mainly localized in nucleus [14, 20]. Consistent with this, we observed nuclear localization of endogenous canine SET and FLAG-tagged canine SET α and β . It has been reported that human SET has nuclear localization signal in its N-terminus regions [20]. Because canine SET γ and δ localize both in cytosol and nucleus, it is possible that C-terminus region of SET is also important for nuclear localization.

Recently, several studies have reported that SET is an attractive drug target for various human tumors [9, 11, 16]. We have previously reported that COG/OP449 (Oncotide Pharmaceuticals, NC, U.S.A.), a peptide antagonist of SET, recovered PP2A activity and exerted anti-tumor effects on canine lymphoma cells [10]. The effects of OP449 were dependent on SET protein levels, which are now named SET α . However, the present study reveals that canine SET β is also a potential PP2A inhibitor. OP449 peptides bind to the C-terminal region of SET, which corresponds to 190–290 of

human SET α [7]. Because SET β does not contain most of this region, it is unclear whether OP449 can antagonize canine SET β functions. This point should be taken into account in future SET-targeting drug development studies in dogs.

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