

# Establishment of a novel neuroblastoma mouse model

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Received June 10, 2008; Accepted August 22, 2008

DOI: 10.3892/ijo\_00000109

**Abstract.** Neuroblastoma is the most common childhood cancer, which arises from sympathetic neural precursors. Because the prognosis of advanced neuroblastoma is known to be poor, developments of new anti-cancer drugs are desperately needed. For screening of therapeutic drugs for neuroblastoma, genetically engineered animal models would be useful. In an attempt to obtain transgenic mice carrying simian virus 40 T-antigen gene under control of tetracycline responsive elements with cytomegalovirus promoter, we found one line of mice exhibiting bilateral adrenal tumors by leakage expression of T-antigen in adrenal gland. These adrenal tumors contained small round tumor cells with increased N/C ratio, showing chromogranin A and neuron specific enolase-like immunoreactivity. By electron microscopy, tumor cells containing neuritic processes with synaptic vesicles surrounding them were observed. The plasma levels of dopamine were significantly elevated in these transgenic mice. MYCN expression levels were significantly elevated in these tumors. These findings indicated that the adrenal tumor was a neuroblastoma. This mouse model would be a useful tool for development of chemotherapeutic drugs and understanding the etiology of neuroblastoma.

## Introduction

Neuroblastoma is a childhood cancer, which arises from sympathetic neural precursors. It is the most common childhood cancer, which accounts for 7-10% of all childhood cancers (1). The median age at diagnosis for neuroblastoma patients is about 18 months with more than 95% of cases detected by 10 years of age (2). Infants with neuroblastoma

tend to present with lower stage disease, and tumors are generally chemosensitive and high cure rates are obtained. But when diagnosed after age of 1 year, these children always have extensive tumors and disseminated metastasis and prognosis has been poor (2). Development of new therapeutic drugs for advanced neuroblastoma is required.

For screening of new drugs for cancers, *in vivo* rodent models are useful in addition to *in vitro* cultured tumor cells. So far, mouse xenograft tumor models have been most widely used. But the results obtained by these xenograft models does not always predict human efficacy (3). Other rodent models are genetically engineered mouse models (transgenic or knockout mice), which became available recently. As for neuroblastoma, Weiss *et al* reported a mouse model of neuroblastoma (4), which may be useful in screening of drugs. But before clinical application, drug testing in as many models as possible is necessary, since efficacies in various mouse models may provide a better assurance for its clinical usefulness. Further development of neuroblastoma mouse models is needed.

Several genetic abnormalities in neuroblastoma have been reported including, ploidy changes, MYCN proto-oncogene amplification deletions of chromosome arms 1p and 11q, and gains of chromosome arm 17q (5). But the etiology of neuroblastoma is not completely understood. To understand the etiology of neuroblastoma, genetically engineered mouse models may be useful.

In this study, we established a novel mouse model of neuroblastoma. This model would be a useful tool for drug screening or investigating the etiology of neuroblastoma.

## Materials and methods

**Animals.** To obtain a transgenic mouse carrying tetracycline inducible simian virus 40 T-antigen (SV40 Tag), a fusion gene comprising tetracycline responsive elements (TRE) with cytomegalovirus promoter and SV40 Tag was designed. The purified fragment (10 µg/ml) was microinjected into the pronucleus of fertilized C57/B6 mice (SLC, Shizuoka, Japan) eggs. The viable eggs were transferred into the oviducts of pseudopregnant female ICR mice (SLC) using standard techniques. Transgenic founder mice were identified by PCR analysis of tail DNAs using the following primers:

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**Key words:** neuroblastoma, transgenic models, simian virus 40

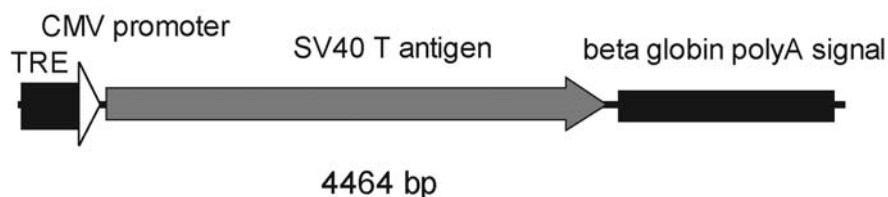


Figure 1. Structure of TRE-SV40 Tag transgene. SV40 Tag is driven by tetracycline responsive elements (TRE) with cytomegalovirus (CMV) promoter.

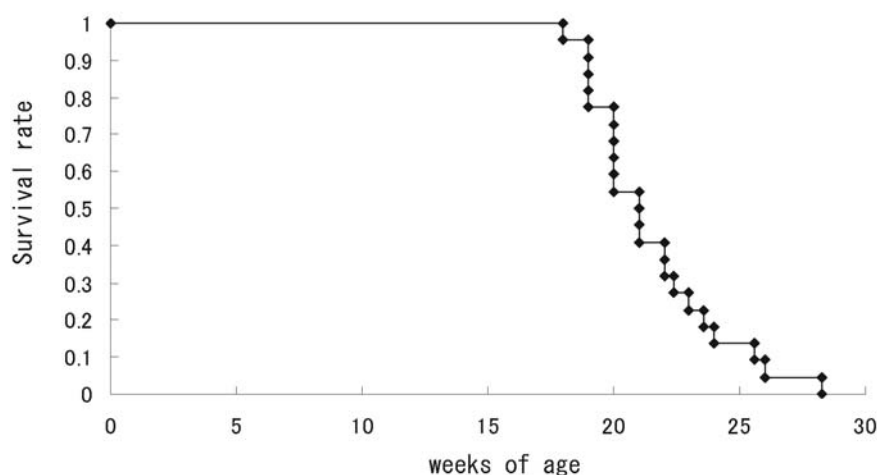


Figure 2. Survival curve of 2-5 line of TRE-SV40 Tag transgenic mice. All transgenic mice died by 28 weeks of age. None of the non-transgenic littermates died by this same age (data not shown).

sense, 5'-GTGTACGGTGGGAGGCCTAT-3'; antisense, 5'-CAGG CACTCCTTTCAAGACC-3'. Transgenic mice were used as heterozygotes. Animals were maintained on standard rodent food (CE-2, 352 kcal/100 g, Japan CLEA, Tokyo, Japan) on a 12-h light/12-h dark cycle. All experimental procedures were approved by the Kyoto University Graduate School of Medicine Committee on Animal Research.

**Immunohistochemistry.** Formalin-fixed, paraffin-embedded tissue sections were immunostained using the avidin-biotin peroxidase complex method (Vectastain 'ABC' Elite kit, Vector Laboratories, Burlingame, CA) as described previously (6). Serial sections were used and the thickness of each section was 5  $\mu$ m. Sections were incubated with anti-chromogranin A antibody (1:500 at final dilution; Dako, Glostrup, Denmark) and neuron-specific enolase (NSE) antibody (1:500 at final dilution; Dako).

**Electron microscope.** Tumor samples were fixed with 2.5% glutaraldehyde at 4°C for overnight. After washing with phosphate buffer, samples were postfixed with 2% OsO<sub>4</sub> at 4°C for 2 h, dehydrated with ethanol, and embedded in Quetol 812 (Nisshin EM, Tokyo, Japan). Ultra-thin sections of samples were cut, stained with uranyl acetate for 15 min and lead acetate for 5 min, and viewed with a H-300 electron microscope (Hitachi, Tokyo, Japan).

**Measurements of serum catecholamines.** Blood was obtained from retro-orbital vein on an ad libitum feeding schedule.

Serum was isolated by centrifugation and stored at -20°C until assayed. Adrenaline, nor-adrenaline and dopamine levels were measured by high performance liquid chromatography at commercial laboratory (SRL, Tokyo, Japan).

**Real-time quantitative RT-PCR.** Total RNA was extracted from adrenal tumor of 14-weeks old male 2-5 line of TRE-SV40 Tag transgenic mice or adrenal gland of their non-transgenic littermates using a Sepasol-RNA kit (Nacalai Tesque, Kyoto, Japan). Reverse transcription (RT) was performed in the presence of random hexamers with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time quantitative PCR was performed using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) using the following primers with power SYBR green: MYCN sense, 5'-CTGCGGTCAGTAGTGTGTC-3', and antisense, 5'-TCGCTCTTGATCTTTTCTG-3', SV40 Tag sense, 5'-AAACACTGCAGGCCAGATTT-3', and antisense, 5'-AAATGAGCCTTGGGACTGTG-3'.

**Statistical analysis.** All values are expressed as means  $\pm$  SE. The statistical significance of the differences in mean values was assessed by Mann-Whitney's U test or Student's t-test as appropriate.

## Results and Discussion

In an attempt to obtain transgenic mice carrying TRE-SV40 Tag (Fig. 1), which does not show tumorigenesis unless it

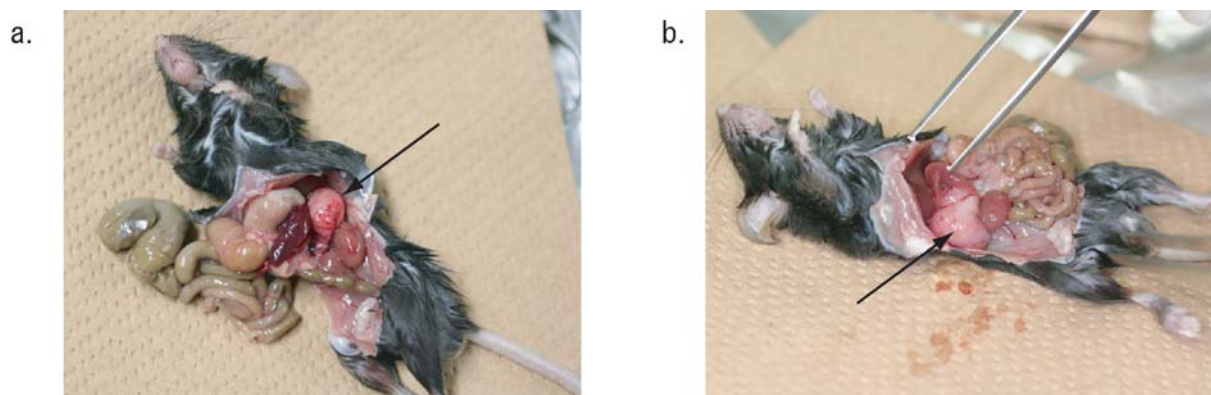


Figure 3. Bilateral adrenal tumors in 2-5 line of TRE-SV40 Tag transgenic mouse. Large bilateral adrenal tumors (arrow) were observed in 2-5 line of TRE-SV40 Tag transgenic mouse. Right adrenal tumor (arrow) involved the liver (b).

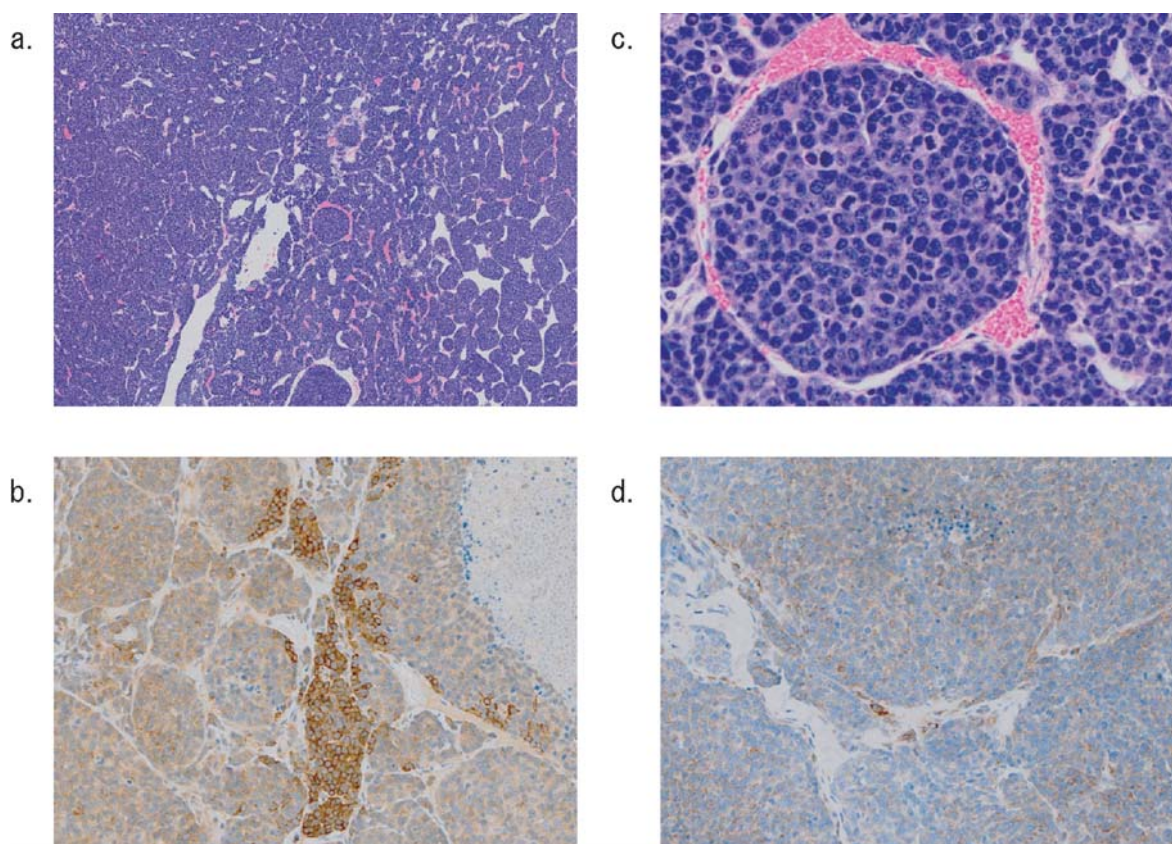


Figure 4. Pathological findings of adrenal tumors. H&E staining of a tumor section (a, x40; b, x400). Tumor sections stained with anti-chromogranin A (c, x200) and anti-NSE antibody (d, x200).

crossed with transgenic mice carrying tet-responsive transcriptional activator (tTA) or reverse-tTA under specific promoter, we unintentionally obtained one line (2-5 line) of TRE-SV40 Tag transgenic mice dying 18-28 weeks of age (Fig. 2). Interestingly, all these mice exhibited bilateral large adrenal tumors (Fig. 3).

By hematoxylin-eosin (HE) staining, these adrenal tumors contained small round tumor cells with increased N/C ratio (Fig 4a and b). These nuclear rich cells seemed highly undifferentiated showing frequent mitosis and necrosis. Rossetti formation was not observed. By immunohisto-

chemical analysis, these cells showed chromogranin A and NSE-like immunoreactivities (Fig. 4c and d). By electron microscopy, cell bodies were evident and neuritic processes were not so evident (Fig. 5a). But, some tumor cells showed neuritic process with synaptic vesicles surrounding them (Fig. 5b). Some tumor cells were rich in neurosecretory dense core granules (Fig. 5c). These pathological findings are consistent with neuroblastoma.

Neuroblastoma secretes various kinds of physiologically active substances including catecholamines. Most of the tumors in our transgenic mice secreted dopamine (non vs.

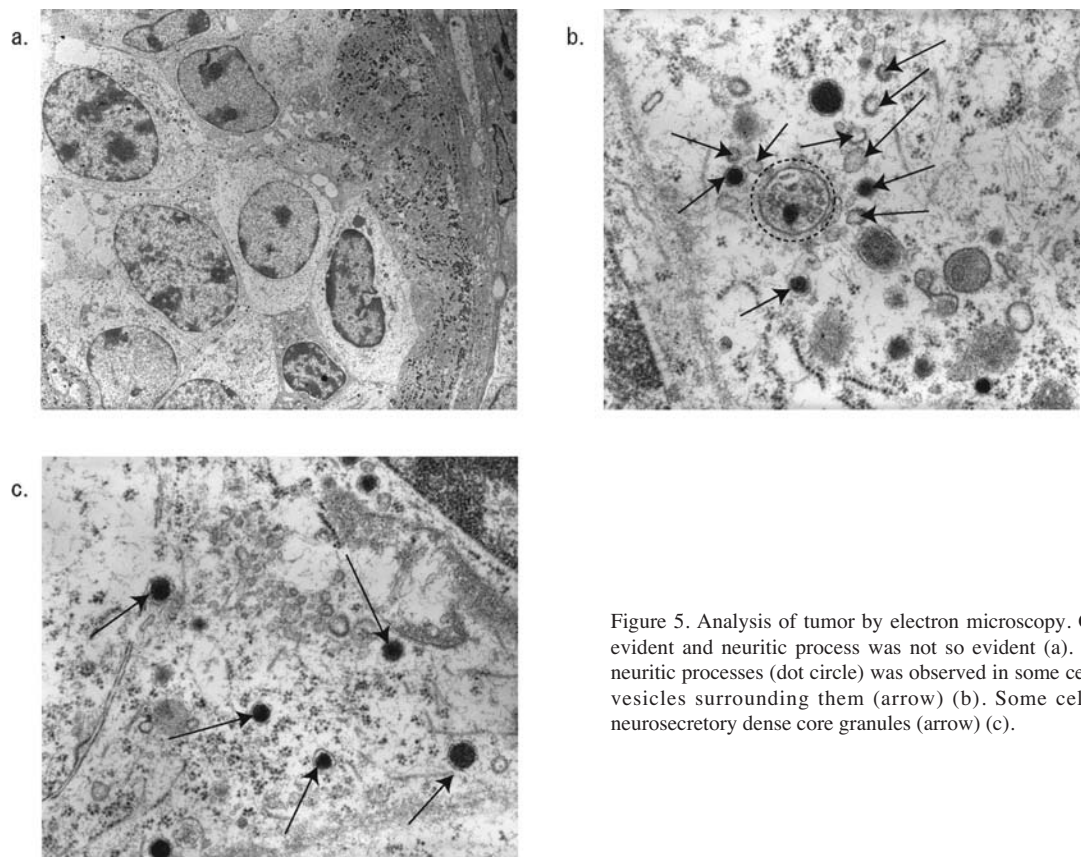


Figure 5. Analysis of tumor by electron microscopy. Cell bodies were evident and neuritic process was not so evident (a). Cross section of neuritic processes (dot circle) was observed in some cells with synaptic vesicles surrounding them (arrow) (b). Some cells were rich in neurosecretory dense core granules (arrow) (c).

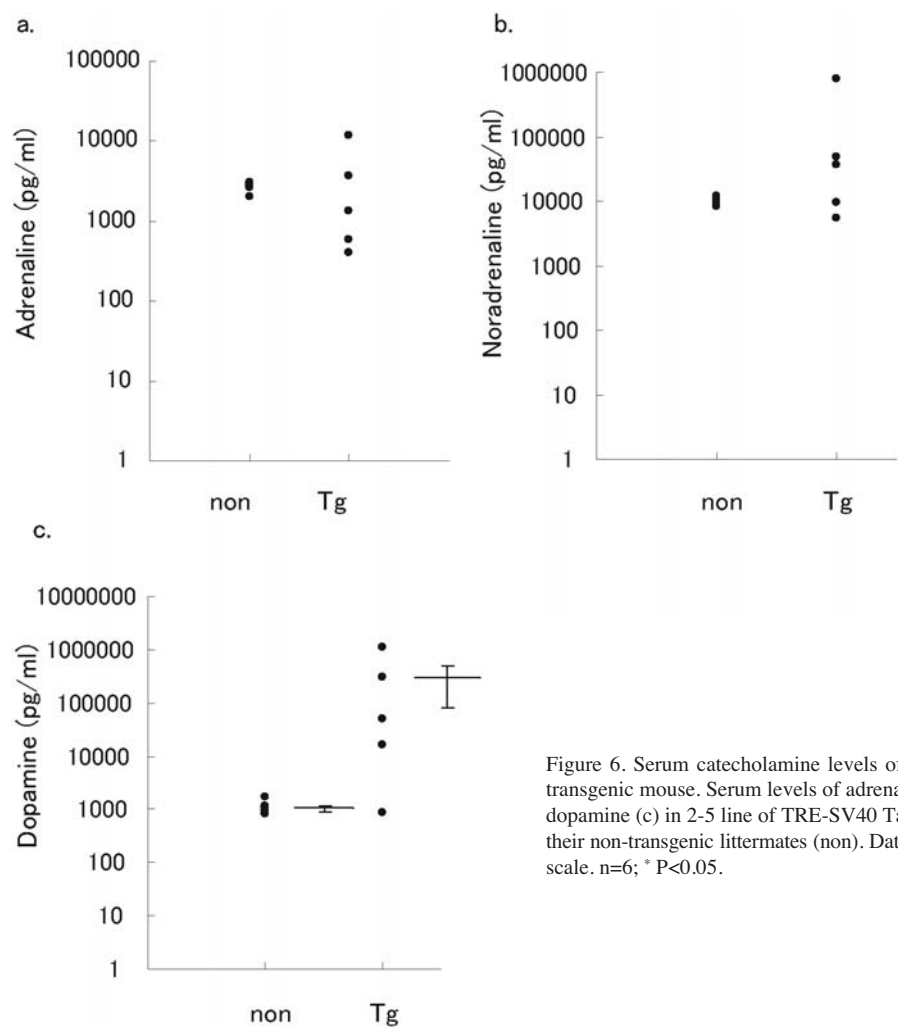


Figure 6. Serum catecholamine levels of 2-5 line of TRE-SV40 Tag transgenic mouse. Serum levels of adrenaline (a), noradrenalin (b) and dopamine (c) in 2-5 line of TRE-SV40 Tag transgenic mouse (Tg) and their non-transgenic littermates (non). Data are presented in logarithmic scale. n=6; \* P<0.05.

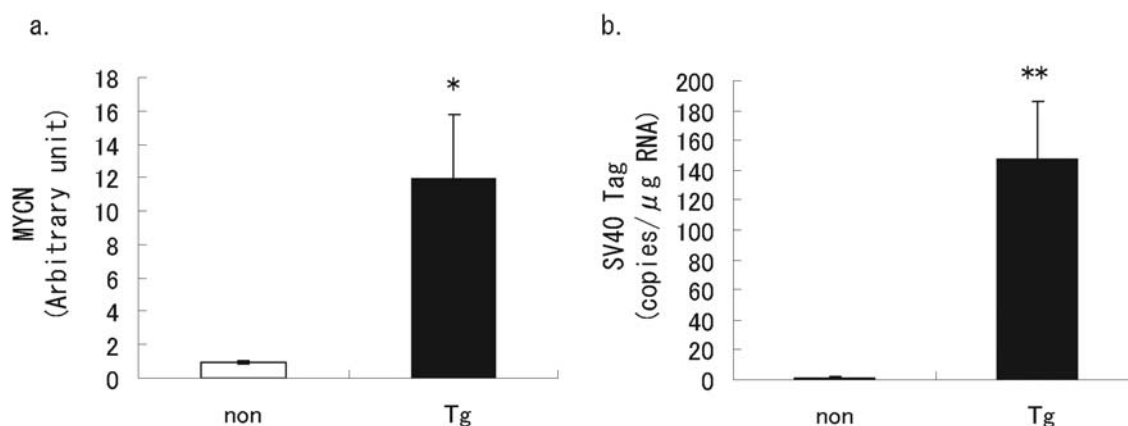


Figure 7. SV40 Tag and MYCN mRNA expression levels in adrenal tumor of 2-5 line of TRE-SV40 Tag transgenic mouse. MYCN (a) and SV40 Tag (b) mRNA expression levels in adrenal tumors of 2-5 line of TRE-SV40 Tag transgenic mice (Tg) and in adrenal gland of their non-transgenic littermates (non). n=7, \*P<0.05; \*\*P<0.01.

Tg;  $1,091.7 \pm 131.9$  vs.  $299,395.8 \pm 213,813.7$  pg/ml,  $P < 0.05$ , n=6), although serum levels are very different from one to another (Fig. 6c). Some secreted noradrenalin (non vs. Tg;  $10,272 \pm 478.5$  vs.  $178,986.2 \pm 154,214.9$  pg/ml,  $P = 0.47$ , n=6) or adrenaline (non vs. Tg;  $2,700.7 \pm 142.1$  vs.  $3,569.4 \pm 2,138.7$  pg/ml,  $P = 0.55$ , n=6). These results also confirm the diagnosis of neuroblastoma.

Amplification of MYCN gene in neuroblastoma has been reported (7), and this correlates with advanced disease (8). The mouse created by Weiss *et al* overexpresses MYCN proto-oncogene in neuroectodermal cells by tyrosine hydroxylase promoter and develops neuroblastoma (4). In our 2-5 line of TRE-SV40 Tag transgenic mice, MYCN mRNA levels were significantly elevated in adrenal tumors (Fig. 7a), which may be caused by leakage expression of SV40 Tag observed in adrenal tumors (Fig. 7b), since SV40 Tag increases the expression of MYCN (9). Presumably, the position of transgene insertion enabled SV40 Tag to express in sympathetic neural precursor cells, which in turn caused MYCN overexpression. Further study is needed to reveal the precise mechanism by which 2-5 line of TRE-SV40 Tag transgenic mice develop adrenal neuroblastoma.

In conclusion, we established a novel mouse model of neuroblastoma. This mouse model would be a useful tool for development of anti-cancer drugs and for understanding the etiology of neuroblastoma.

## Acknowledgements

We thank Chieko Ishimoto and Chinami Shiraiwa for excellent technical assistance. This study was supported by funds from the Ministry of Education, Science, Culture, Sports, Science and Technology of Japan, and the Ministry of Health, Labour and Welfare of Japan.

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