

Cross-Species Studies on Tumor Biology in Companion Animals

(伴侶動物の腫瘍生物学に関する種横断的な研究)

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GENERAL INTRODUCTION

Advances in veterinary medicine have expanded the opportunities to diagnose and treat neoplastic diseases in animals. At the same time, the characterization of disease profiles across a broad range of species has been facilitated by the increasing number of companion animals, including dogs and cats as well as other exotic animals, and by the development of *ex situ* conservation program for wildlife in zoos and aquariums. As a result, neoplastic diseases have now been documented in many animal species in the worldwide, and clinical issues in veterinary and comparative oncology are rapidly emerging.

Comparative oncology deepens our understanding of tumor biology across species by integrating perspectives from ecology, physiology, and molecular biology. Thereby providing new insights into tumor development and tumor-suppressive strategies across species. Such cross-species approaches also hold the potential to advance the development of novel therapeutic strategies and to enhance our understanding of tumor mechanisms in human medicine.

In this thesis, I focused on dogs, which exhibit a high incidence of tumors and are widely kept as companion animals, and the African pygmy hedgehog (*Atelerix albiventris*), a small exotic pet species known for its high tumor susceptibility and increasing popularity in captivity. My overall goal is to address clinically relevant issues

that have arisen in veterinary practice and to contribute to the development of comparative oncology across species.

First, I investigated DNA mismatch repair deficiency (dMMR) and microsatellite instability (MSI) in canine tumors. The MMR system is regulated mainly by four genes: mutS homologue 2 (*MSH2*), mutS homologue 6 (*MSH6*), mutL homologue 1 (*MLH1*), and postmeiotic segregation increased 2 (*PMS2*), and plays key roles in preserving genomic stability by recognizing and repairing mismatched nucleotides during DNA replication. Microsatellites (MS) are short repetitive sequences composed of 1–6 base pairs that are dispersed throughout non-coding regions of the genome (Tautz, 1993). MSI is a condition in which replication errors, such as insertions and deletions, accumulate in MS loci and cause differences in repeat length between matched normal and tumor tissues. It is now known that dMMR induces MSI and increases the somatic mutation burden, ultimately promoting tumorigenesis (Eshleman et al., 1995; Eshleman and Markowitz, 1996; Umar et al., 1997).

In human oncology, dMMR and MSI are routinely assessed by immunohistochemistry (IHC) and PCR-based methods to elucidate genetic mechanisms of carcinogenesis (Richman, 2015). Numerous reports have shown that tumors with dMMR and MSI are highly responsive to immune checkpoint inhibitors (ICIs) (Le et al.,

2015; Kumar et al., 2017; Coventry et al., 2019), and thus dMMR/MSI has been established as a predictive biomarker for ICI efficacy (Yi et al., 2018). Recently, clinical trials of immune checkpoint blockade therapies using PD-1 or PD-L1 inhibitors have been reported in dogs with spontaneous tumors (Maekawa et al., 2017; Igase et al., 2020c, 2024). In these studies, the objective response rate was approximately 20%, although cases numbers were limited. Therefore, identifying predictive markers of treatment response is essential for the effective application of immune checkpoint blockade therapies in canine oncology.

In Chapter 1, I aimed to assess the cross-reactivity of antibodies against human MMR proteins with canine MMR proteins and to evaluate the MMR protein expression patterns in canine tumors by IHC, in order to identify tumor types exhibiting dMMR. In Chapter 2, I examined MSI status in several types of canine tumors to identify tumor types that frequently harbor MSI. Furthermore, I evaluated its relationship between MMR and dMMR in clinical cases and cell lines. Based on these data, I selected MS loci that are most suitable for MSI analysis in dogs, with the aim of improving the efficacy and reliability of MSI analysis in veterinary practice.

Next, I focused on KIT, a receptor tyrosine kinase that is highly expressed in canine cutaneous mast cell tumors (MCTs) and gastrointestinal stromal tumors (GISTs)

in dogs. Receptor tyrosine kinase KIT plays an essential role in cell proliferation and differentiation. Activating mutations in the *c-kit* gene, which encodes KIT, induce ligand-independent autophosphorylation and drive tumorigenesis in both humans and dogs.

In canine MCTs and GISTs, IHC for KIT reveals distinct staining patterns among cases. KIT expression is typically categorized into three patterns: pattern I, membranous staining; pattern II, paranuclear focal staining; and pattern III, diffuse cytoplasmic staining. These staining patterns correlate with clinical behavior, with increasing risks of metastasis, recurrence, and shorter survival from pattern I to pattern III (Kiupel et al., 2004; Thompson et al., 2011; Brocks et al., 2020). It has been hypothesized that such variation in KIT staining reflects aberrant subcellular localization caused by *c-kit* mutations, and numerous studies have investigated the relationship between KIT localization abnormalities and *c-kit* mutations. However, the findings have been inconsistent (Webster et al., 2006; Thompson et al., 2011; Chen et al., 2022), and a definitive causal relationship has not been established. These inconsistencies may be partly explained by the fact that most previous studies relied on IHC analysis of clinical samples, which is limited in its ability to resolve subcellular localization at single-cell resolution. Furthermore, molecular studies that directly evaluate the functional consequences of *c-kit* mutations *in vitro* remain scarce. Therefore, in Chapter 3, I

introduced canine *c-kit* mutations into cultured cells to characterize the subcellular localization of each KIT mutants at single-cell resolution and to determine how differences in subcellular localization influence downstream signaling, cell proliferation, and cell survival.

Finally, I addressed Peto's paradox, which describes the observation that tumor incidence does not scale in a simple linear fashion with body sizes and lifespan across species (Peto et al., 1975; Peto, 2016). Large, long-lived animals such as whales and elephants possess far more cells and undergo many more lifetime cell divisions than small and/or short-lived animals, yet they do not exhibit proportionally higher tumor rates (Abegglen et al., 2015; Nunney et al., 2015; Boddy et al., 2020). many studies on Peto's paradox have focused on large and/or long-lived animals and have revealed tumor-suppressive mechanisms that appear to have evolved in these species (Seluanov et al., 2009; Abegglen et al., 2015; Keane et al., 2015). In contrast, studies that directly address tumorigenesis in small and/or short-lived animals remain extremely limited.

The African pygmy hedgehog (*Atelerix albiventris*) is one of the most popular exotic pets in many countries, including Japan, and is characterized by a high incidence of neoplastic disease. In several necropsy studies, 29%–60% of African pygmy hedgehogs were diagnosed with neoplastic disease (Raymond and White, 1999; Raymond

and Garner, 2001; Okada et al., 2018), and 74.6% of these tumors were classified as malignant (Okada et al., 2018). Both endogenous factors determining tumor susceptibility and exogenous environmental factors are likely to contribute to this high tumor burden (Ames, 1989; Hussain and Harris, 2000), yet it remains unclear why tumors develop so frequently in this small-bodied, relatively short-lived species. One major limitation is the lack of basic research resources for this species, including established cell lines and comprehensive genome information. Thus, establishing these fundamental resources is a prerequisite for advancing research on neoplastic diseases in African pygmy hedgehogs.

In Chapter 4, I established a new cell line derived from histiocytic sarcoma (HS) in an African pygmy hedgehog. In this species, HS typically arises in various organs, including the skin, abdominal organs, and nervous system, similar to canine HS (Ogihara et al., 2016, 2017; Koizumi and Kondo, 2019; Koizumi et al., 2020). To date, chemotherapy protocols have not been established for HS or other tumors in African pygmy hedgehogs, and cases with systemic metastases cannot be cured by surgical resection alone and therefore a poor prognosis. I used this newly established cell line to screen for novel anti-tumor drugs and to analyze their mechanisms of action, with the aim of improving treatment options for this species.

In Chapter 5, I analyzed the draft genome of African pygmy hedgehogs and identified candidate genomic features that may represent endogenous factors contributing to its high tumor incidence. I then evaluated, *in vitro*, how these candidate features affect tumor-suppressive pathways, in order to clarify how species-specific features of African pygmy hedgehogs may predispose them to tumor development.

Taken together, my dissertation addresses clinically relevant issues in veterinary oncology through reverse-translational and comparative approaches. This work emphasizes the importance of developing oncology not only for dogs and cats, but also for the broader diversity of animal species encountered in modern veterinary practice.

Chapter 1

Immunohistochemical analysis of mismatch repair deficiency
in canine tumors

SUMMARY

The DNA mismatch repair (MMR) system preserves genomic stability by identifying and repairing mismatched nucleotides in the DNA replication process. The dysfunction of the MMR system, also known as mismatch repair deficiency (dMMR), is implicated as a predictive biomarker for the efficacy of immune checkpoint blockade therapy regardless of the tumor type in humans. This study aimed to evaluate the immunolabeling of MMR proteins in canine tumors and to identify the types of tumors having dMMR. First, I performed immunohistochemistry in 8 different canine tumors (oral malignant melanoma, high-to-intermediate grade lymphoma, mast cell tumor, malignant mammary gland tumor, urothelial carcinoma, hepatocellular carcinoma, osteosarcoma, and hemangiosarcoma) with 15 samples each to analyze the immunolabeling of canine mismatch repair proteins (MSH2, MSH6, and MLH1) using anti-human monoclonal antibodies. I found that more than half of canine oral malignant melanoma (60%) and hepatocellular carcinoma (53%) samples and fewer of the other canine tumors had loss of immunolabeling in ≥ 1 MMR protein (i.e., evidence of defective MMR proteins, based on the definition of dMMR in the humans). Antibodies against human MSH2, MSH6, and MLH1 were cross-reactive with the corresponding protein in the dog as confirmed using MMR gene knockout canine cell lines. Further studies are

required to investigate the clinical outcomes in canine spontaneous tumors with dMMR to determine the potential for immune checkpoint blockade therapy for these tumor types.

INTRODUCTION

The DNA mismatch repair (MMR) system plays key roles in preserving genomic stability by identifying and repairing mismatched nucleotides in the DNA replication process. The MMR system is regulated mainly by four genes: mutS homologue 2 (*MSH2*), mutS homologue 6 (*MSH6*), mutL homologue 1 (*MLH1*), and postmeiotic segregation increased 2 (*PMS2*). These genes were first found as causative germline mutation in patients with Lynch syndrome in 1993 (Leach et al., 1993; Bronner et al., 1994; Fishel et al., 1994). Lynch syndrome is a hereditary disease and patients with this disease are prone to tumors, particularly colorectal cancer and endometrial cancer. In addition to Lynch syndrome, defects of the MMR system are also caused by somatic mutation or hypermethylation of promoter sequences and has been found in various sporadic tumors (McGivern et al., 2004; Mensenkamp et al., 2014). It is now known that MMR deficiency (dMMR) induces microsatellite instability and increases mutation rates, leading to tumor development (Eshleman et al., 1995; Eshleman and Markowitz, 1996; Umar et al., 1997).

In humans, immunohistochemical analysis for MSH2, MSH6, MLH1, and PMS2 proteins are used for detection of dMMR (Richman, 2015). Several reports have shown that tumors with dMMR and/or microsatellite instability-high are sensitive to immune checkpoint blockade therapy, particularly PD-1 inhibitor therapy (Kumar et al., 2017; Yi

et al., 2018; Zhao et al., 2019). For instance, Le *et al.*, showed that the objective response rate in MMR-deficient colorectal cancer or MMR-proficient colorectal cancer were 40% and 0%, respectively (Le et al., 2015). Therefore, dMMR is implicated as a predictive biomarker for efficacy of immune checkpoint blockade therapy regardless of tumor type.

In dogs, accumulation of mutations may be associated with development of tumors. However, there are few reports about microsatellite instability and dMMR in canine cancers. A previous study confirmed using immunohistochemistry (IHC) that MLH1, MSH2, and MSH6 proteins were expressed in 44 mast cell tumor samples (Munday et al., 2009). In a case report on two dogs with colon adenocarcinoma, nuclei of tumor cells in both dogs were positively labeled with IHC for MLH1 protein (Makovický et al., 2016). However, there are no reports evaluating the defects of the above four MMR proteins in other types of canine tumors. In addition, two recent clinical studies of immune checkpoint blockade therapies using PD-1 or PD-L1 inhibitors were reported for spontaneous tumors in dogs (Maekawa et al., 2017; Igase et al., 2020c). In these studies, tumor response rates were approximately 20%, although the number of cases were limited. Thus, searching for predictive markers for treatment response is essential for the effective use of immune checkpoint blockade therapies. Establishment of methods to analyze MMR proteins expression and identification of tumors with dMMR in canine

cancer are important for promoting immune checkpoint blockade therapies in the future.

This study aimed to evaluate the immunolabeling for MMR proteins in canine tumors and to identify the types of tumors having dMMR. Also, cross-reactivity of antibodies against human MMR proteins to canine MMR proteins was confirmed with MMR gene knockout cell lines.

MATERIALS & METHODS

Cell culture

Human embryonic kidney (HEK) 293T cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM)-based complete medium (DMEM mixed with 10% fetal bovine serum (FBS), 100 units/ml penicillin-streptomycin and 55 μ M 2-mercaptoethanol). CIP-p (canine mammary gland tumor) cell line (Nakagawa et al., 2006), kindly provided by Dr. Takayuki Nakagawa (The University of Tokyo), was cultured in RPMI1640-based complete medium (RPMI1640 mixed with 10% FBS, 100 units/ml penicillin-streptomycin and 55 μ M 2-mercaptoethanol). All cell lines were maintained at 37°C in a humidified CO₂ gas incubator.

Lentiviral transduction of knockout vectors

According to the previous reports (Igase et al., 2019), CRISPR-Cas9 small guide RNAs (sgRNA), which targets MMR genes (*mlh1*, *msh2*, and *msh6*), were constructed with online CRISPR design tool (<http://zlab.bio/guide-design-resources>). The sequences of sgRNAs of canine MLH1, canine MSH2, and canine MSH6 were GTGGTGAACCGCATCGCGGC, GCGCTTCTTCCAGGCCATGC, and GAAGTAAGGCCTAAGGTCCA, respectively. These oligonucleotides were

synthesized and inserted separately into lentiCRISPR v2 plasmid, which was donated by Dr. Feng Zhang (Addgene, Cambridge, MA, #52961; <http://n2t.net/addgene:52961>; RRID: Addgene 52961). To produce lentivirus-expressing sgRNAs that target each gene, HEK293T cells were seeded at 3.0×10^5 cells/well onto a 6-well plate a day before the transfection. On the day of transfection, 1.25 µg of each plasmid was mixed with PEI Max (Polysciences inc., Warrington, PA) in OPTI-MEM buffer (Thermo Fisher Scientific K.K., Tokyo, Japan), incubated for 15 min at RT, and added onto HEK293T cells. Twenty-four hours after transfection, supernatant was replaced with RPMI1640-based complete medium without antibiotics. After another 24 hours, the supernatant containing lentiviruses was collected and transferred onto CIP-p cells. Twelve hours after infection, the supernatant was replaced with RPMI1640-based complete medium, followed by addition of 7.5 µg/ml of puromycin to select stably transduced cells. Individual single clones were isolated by limited dilution method and further analyzed by western blotting to confirm the knockout of each protein.

Western blotting analysis

Each cell line was collected and lysed with NP-40 lysis buffer. Equal amounts of protein samples (30 µg) were electrophoresed in 7% acrylamide gel before transferring

onto membranes. The membranes were incubated with each primary antibody (the same antibodies as used in IHC) diluted at 1:1,000 in 0.5% skimmed milk in TBS-T overnight at 4°C. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (Merck Millipore, Darmstadt, Germany) or HRP-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch, Philadelphia, PA) was used as a secondary antibody. The membranes were soaked in Western Lightning Plus-ECL (PerkinElmer, Tokyo, Japan) to visualize the immunoreaction. Immunoreactive bands were quantified and analyzed with AMERSHAM Image Quant 800 (Global Life Sciences Technologies Japan, Tokyo, Japan). The membranes were reprobed with mouse monoclonal anti-beta actin antibody (Sigma-Aldrich Japan K.K., Tokyo, Japan) to verify equal loading of proteins in each lane.

Immunohistochemistry (IHC)

Formalin-fixed and paraffin-embedded (FFPE) canine tumor samples and testis tissue sample from a healthy dog were collected from diagnostic pathology laboratories (Yamaguchi University, Veterinary Pathology Diagnostic Center, Fukuoka, Japan; and IDEXX Laboratories, Tokyo, Japan). All samples were diagnosed by the veterinary pathologists in each laboratory. Fifteen samples were used for each tumor type (oral

malignant melanoma, lymphoma, mast cell tumor, malignant mammary gland tumor, urothelial carcinoma, hepatocellular carcinoma, osteosarcoma, and hemangiosarcoma).

Sections were cut at 5 μ m thickness and mounted on charged MAS-coated glass slides (Matsunami Glass, Osaka, Japan). After deparaffinization and rehydration, antigen retrieval was performed in Dako Target Retrieval Solution pH9 (Agilent Technologies Japan, Ltd., Tokyo, Japan). The sections were incubated in 3% hydrogen peroxide in PBS to block endogenous peroxidase activity. To block non-specific protein activity, the sections were incubated in 5% skimmed milk and 5% bovine serum albumin in PBS. Next, the sections were incubated with mouse monoclonal anti-human MLH1 antibody (G168-15, GeneTex, Inc., Irvine, CA; 1:50 dilution), rabbit monoclonal anti-human MSH2 antibody (SP46, Abcam, Cambridge, U.K; 1:100 dilution), mouse monoclonal anti-human PMS2 antibody (163C1251, Novus biologicals, LLC, Littleton, CO; 1:250 dilution), mouse monoclonal anti-human PMS2 antibody (1G4E6, Proteintech Group, Inc., Rosemont, IL; 1:200 dilution) or mouse monoclonal anti-human MSH6 antibody (44/MSH6, BD Transduction Laboratories, San Jose, CA; 1:250 dilution). Isotype-matched antibodies, anti-rabbit IgG antibody (DA1E, Cell Signaling Technology, Inc., Danvers, MA) and anti-mouse IgG1 antibody (P3.6.2.8.1, eBioscience, Inc., San Diego, CA), were used as negative immunolabeling control. The sections were incubated with

Histofine Simple Stain mouse MAX PO (Nichirei Bioscience Inc., Tokyo, Japan) or Histofine Simple Stain rabbit MAX PO (Nichirei Bioscience Inc.). Peroxidase Stain DAB Kit (Nacalai Tesque, Inc., Kyoto, Japan) was used to visualize the immunoreaction. The sections were counterstained with Mayer's hematoxylin, dehydrated in alcohol, cleared in xylene, and mounted with xylene-based mounting solution. To bleach melanin granules, OMM samples were incubated in 10% hydrogen peroxide in PBS for 10 min at 65°C before the antigen retrieval. After incubation with the secondary antibody, Simple Stain AEC Solution (Nichirei Bioscience Inc.) was used to visualize the immunoreaction instead of DAB. Finally, after counterstaining with Mayer's hematoxylin, these were mounted with water-soluble mounting solution. Immunoreactivity was considered positive if cell nuclei in the tumor cells were immunolabeled with each antibody as compared with the isotype control. I simultaneously evaluated the immunolabeling of the normal tissue area of each sample as a positive control. Immunolabeling was considered negative when the nuclei of tumor cells showed negative immunolabeling. Immunoreactivity in each sample was independently evaluated by four observers and dMMR was defined as a loss of immunolabeling in ≥ 1 MMR protein, according to the criteria used for humans (Richman, 2015).

RESULTS

Immunohistochemistry of MMR proteins in various canine tumor samples.

Firstly, I used a normal canine testis tissue to confirm the immunolabeling of each antibody. Both of the anti-human PMS2 antibodies showed negative immunolabeling. Due to this result, the expression of three MMR proteins—MLH1, MSH2, and MSH6—were analyzed in canine tumors in this study.

Immunohistochemistry was performed on a total of 120 FFPE samples of eight different types of canine tumors (15 samples of each tumor; Table I-1).

Representative results of immunostaining are shown in Figure I-1. The number of samples showing negative immunolabeling for each MMR protein and number of dMMR samples in the total sample are summarized in Figure I-2 and Table I-1. The proportion of tumor samples with evidence of dMMR (i.e. negative immunolabeling for ≥ 1 MMR protein) was 9/15 (60%) for oral malignant melanoma, 8/15 (53%) for hepatocellular carcinoma, 4/15 (27%) for lymphoma, 2/15 (13%) for mammary gland tumor, and 1/15 (7%) for osteosarcoma. None of the mast cell tumor, urothelial carcinoma, and hemangiosarcoma samples had evidence of dMMR (i.e. all samples had positive nuclear immunolabeling for each of the 3 MMR proteins). Negative immunolabeling was more frequent for MLH1 and MSH6, and less frequent for MSH2

(Figure I-2).

Negative immunolabeling for ≥ 2 MMR proteins was observed for 4/15 samples of oral malignant melanoma, 5/15 samples of hepatocellular carcinoma, and 1/15 samples of osteosarcoma (Table I-2). In the 4 oral malignant melanoma samples, no MMR protein was detected in two samples and only MSH2 protein was detected in 2 other samples. In the 5 hepatocellular carcinoma samples, no MMR protein was detected in three samples and only MSH2 protein was detected in 2 other samples. In the 1 osteosarcoma sample, no MMR proteins were observed (Table I-2).

Confirmation of cross-reactivity of antibodies against each MMR protein

To confirm the binding of antibodies against canine MMR proteins (MLH1, MSH2, and MSH6), I established MMR gene knockout cell lines using a CRISPR/Cas9 system. Firstly, I confirmed that all three MMR proteins were detected in CIP-p cell line by western blotting (data not shown). Each antibody was found to cross-react with each canine MMR protein, as the expression of each MMR protein was lost in the cell line with knockout of the corresponding MMR gene (Figure I-3). In addition, immunolabeling for MSH6 protein was lost when the *msh2* gene was knocked out in CIP-p cells, and

immunolabeling for MSH2 protein was lost when the *msh6* gene was knocked out in CIP-
p cells.

DISCUSSION

This is the first study to analyze deficiency of the MMR system in multiple types of canine tumors. Although I used human monoclonal antibodies to analyze dMMR, I found that these antibodies specifically reacted with canine MMR proteins, except PMS2. In the immunohistochemical analysis, dMMR tended to vary among canine tumor types. None of the mast cell tumor, urothelial carcinoma, and hemangiosarcoma samples showed a dMMR system, while oral malignant melanoma and hepatocellular carcinoma tended to have dMMR. These results on mast cell tumor were consistent with those of a previous study (Munday et al., 2009); however, there are no reports on dMMR in other types of tumors in veterinary medicine.

In human malignant melanoma, a previous report (Korabiowska et al., 2000) showed a strong tendency of dMMR (approximately 55%), which is consistent with my result on oral malignant melanoma. Tumors in humans with dMMR are associated with better response of immune checkpoint blockade (Le et al., 2015, 2017). In canine clinical studies (Maekawa et al., 2017; Igase et al., 2020c), PD-1 or PD-L1 inhibitor therapy seemed to be effective in some cases with oral malignant melanoma, suggesting that tumors with dMMR are possibly involved in the response to immune checkpoint blockade therapy in dogs.

In human hepatocellular carcinoma, the proportion of dMMR was reported approximately 3% (Le et al., 2017). Therefore, it was unexpected that canine hepatocellular carcinoma was strongly prone to a defective MMR system. Human hepatocellular carcinoma is mainly caused by hepatitis B and C virus infection (El-Serag, 2012), whereas canine hepatocellular carcinoma is generally not caused by viral infection. Thus, the nature of HCC may be different between dogs and humans. These results indicated that hepatocellular carcinoma might be a good candidate for the treatment of PD-1 or PD-L1 inhibitors in a future study. In addition, tumors with dMMR are known to acquire resistance to some anticancer drugs in human medicine (Aebi et al., 1996, 1997; Fedier et al., 2001), suggesting that dMMR may be related to resistance to chemotherapy that typifies canine hepatocellular carcinoma and oral malignant melanoma.

In humans, it is known that MSH6 protein functions as a heterodimeric complex with MSH2 protein and is degraded in the absence of MSH2 protein (Zhao et al., 2019). These IHC results supported this phenomenon because tumor samples with loss of MSH2 protein commonly showed negative immunolabeling for MSH6. However, the reverse was not necessarily true. This may be because MSH2 protein was able to form a complex with MSH3 protein in addition to MSH6 protein (Zhao et al., 2019). Results of the knockout cell lines experiment also showed that the loss of MSH2 protein expression was

accompanied with a defect of MSH6 protein (Figure I-3). However, our data also showed that loss of MSH2 protein was observed in the *msh6*-knockout cell line. Furthermore, the tumor samples with loss of MSH2 protein immunolabeling was also accompanied with a defect of MLH1 protein in this study. Therefore, MSH2 and MSH6 proteins in dogs may have a function/mechanism that is different from the human proteins; thus, further analysis is needed.

In the present study, there are several limitations. First, 15 samples of tumor types were used in this survey by may be insufficient to definitively identify the frequency of dMMR in each canine tumor types. Second⁴ MMR proteins were used for dMMR analysis in humans, but I could not examine the expression of PMS2 protein due to the lack of a specific antibody against canine PMS2. Moreover, several other MMR proteins are also known in humans (Habraken et al., 1996; Palombo et al., 1996; Zhao et al., 2018), and it is possible that other proteins function as major MMR proteins in dogs. Third, I analyzed the immunolabeling of three MMR proteins in this study, but detecting positive immunostaining may not always relate to the functionality of the protein. Fourth, the clinical outcome in canine tumors with dMMR was not evaluated in this study. All these points are essential to be considered in a future study.

In conclusion, I found that canine oral malignant melanoma and hepatocellular carcinoma samples were prone to defective MMR proteins. Further studies to analyze microsatellite instability and to elucidate the relationship between response to immune checkpoint blockade therapy and canine tumors with dMMR are expected.

(A)

(B)

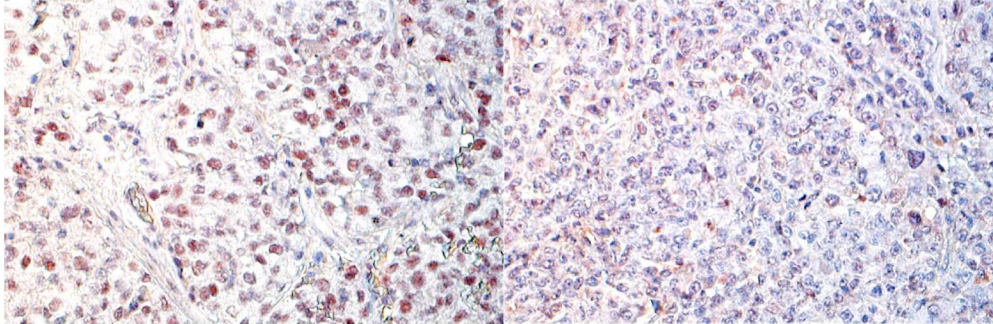


Figure I-1. Oral malignant melanoma, dog. Immunohistochemistry. This case has positive immunolabeling for mutS homologue 2 (MSH2) protein (A), but negative nuclear immunolabeling for mutL homologue 1 (MLH1) protein (not shown) and mutS homologue 6 (MSH6) protein (B). Thus, the MMR system in this sample is considered deficient.

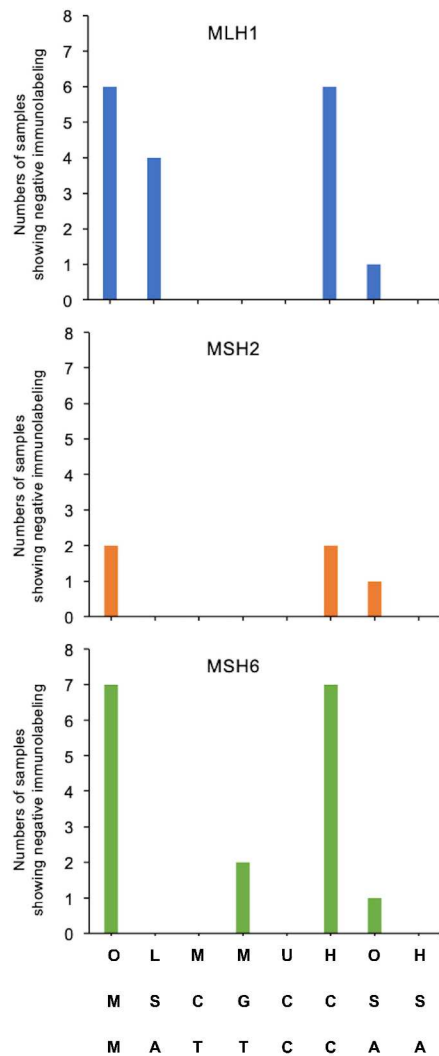


Figure I-2. Number of samples (from 15 total for each tumor type) showing negative immunolabeling for each MMR protein (MLH1, MSH2, and MSH6). Negative immunolabeling was more frequent in canine OMM and HCC than in other tumors. Negative immunolabeling was more frequent for MLH1 and MSH6 than for MSH2. OMM, oral malignant melanoma; LSA, lymphoma; MCT, mast cell tumor; MGT, mammary gland tumor; UCC, urothelial cell carcinoma; HCC, hepatocellular carcinoma; OSA, osteosarcoma; HAS, hemangiosarcoma.

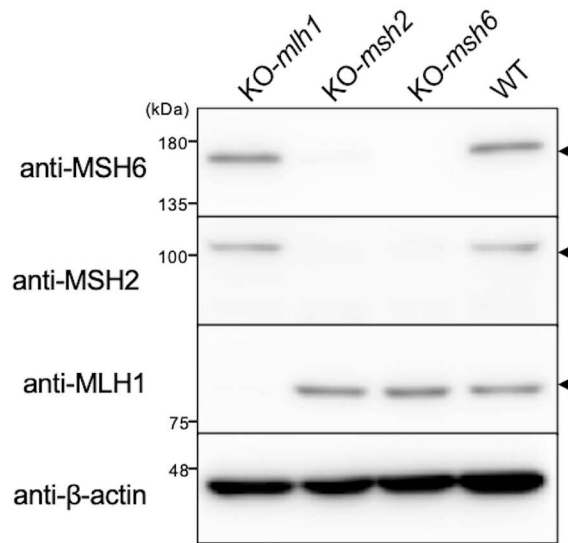


Figure I-3. Validation of antibodies used for immunohistochemistry. Whole cell lysates were prepared from CIP-p cell lines of wild type or with knockout of canine mismatch repair genes *mlh1*, *msh2*, and *msh6*. Proteins were separated with SDS-PAGE, followed by western blotting with anti-human MLH1 antibody, anti-human MSH2 antibody, or anti-human MSH6 antibody. β-actin was used as the protein loading control. These results support that these antibodies specifically reacted with canine MMR proteins.

Table I-1. Evidence of mismatch repair deficiency (dMMR) in 8 types of canine tumors.

Tumor type	Details	Number of samples	Numbers of samples with negative immunolabeling			dMMR ^a	
			MLH1	MSH2	MSH6	Total	
Oral malignant melanoma	Melanotic	14	6	2	7	9	9/15 (60%)
	Amelanotic	1	0	0	0	0	
Lymphoma	T cell	8	3	0	0	3	4/15 (26%)
	B cell	5	0	0	0	0	
Mast cell tumor	non-T non-B	2	1	0	0	1	
	High grade ^b	8	0	0	0	0	0/15 (0%)
Mammary gland tumor	Low grade ^b	7	0	0	0	0	
	Simple carcinoma	13	0	0	1	1	2/15 (13%)
Urothelial carcinoma	Complex carcinoma	2	0	0	1	1	
	Urinary bladder to urethra	15	0	0	0	0	0/15 (0%)
Hepatocellular carcinoma	Poorly differentiated	7	2	1	3	4	8/15 (53%)
	Moderately differentiated	2	1	0	1	1	
Osteosarcoma	Well-differentiated	6	3	1	3	3	
	Appendicular skeleton	12	1	1	1	1	1/15 (7%)
Hemangiosarcoma	Others	3	0	0	0	0	
	Spleen	13	0	0	0	0	0/15 (0%)
	Others	2	0	0	0	0	

^adMMR was defined as a loss of immunostaining in ≥ 1 mismatch repair protein.

^bKiupel histologic grading system.

Table I-2. The samples showing negative immunolabeling for ≥ 2 MMR proteins.

Tumor type	MLH1	MSH2	MSH6
Oral malignant melanoma ($n = 4$)	—	—	—
	—	—	—
	—	+	—
	—	+	—
Hepatocellular carcinoma ($n = 5$)	—	—	—
	—	—	—
	—	—	—
	—	+	—
Osteosarcoma ($n = 1$)	—	+	—
	—	—	—

+, positive immunostaining; —, negative immunostaining.

Chapter 2

Relationship of microsatellite instability to mismatch repair deficiency in
canine tumors

SUMMARY

Background: Microsatellite instability (MSI) is a type of genomic instability caused by mismatch repair deficiency (dMMR) in tumors. Studies on dMMR/MSI are limited, and the relationship between dMMR and MSI is unknown in tumors of dogs.

Objectives: I aimed to identify the frequency of dMMR/MSI by tumor type and evaluate the relationship between dMMR and MSI in tumors of dogs.

Animals: In total, 101 dogs with 11 types of malignant tumors were included.

Methods: I extracted DNA from fresh normal and tumor tissues. Twelve microsatellite loci from both normal and tumor DNA were amplified by PCR and detected using capillary electrophoresis. Each microsatellite was defined as MSI if a difference in product size between the tumor and normal DNA was detected. The dMMR was evaluated by immunohistochemistry using formalin-fixed paraffin-embedded tumor tissues. Next, I confirmed whether dMMR induces MSI by serial passaging of MMR gene knockout cell lines for 3 months.

Results: Microsatellite instability was detected frequently in oral malignant melanoma. The number of MSI-positive markers was higher in cases with dMMR than in those with proficient MMR ($p < .0001$). Statistical analysis indicated that the occurrence of MSI in

FH2305 might have relevance to dMMR. Furthermore, MSI occurred in dMMR cell lines 3 months after passaging.

Conclusions and clinical importance: Microsatellite instability and dMMR more frequently were found in oral malignant melanoma than in other tumors, and dMMR has relevance to MSI in both clinical cases and cell lines.

INTRODUCTION

Microsatellites (MS) are short repetitive sequences composed of 1–6 base pairs dispersed throughout non-coding regions in the genome (Tautz, 1993). Microsatellite instability (MSI) is a condition in which replication errors, such as insertions and deletions, accumulate in MS and cause differences in MS repeats between normal and tumor tissues. In humans, MSI is detected in some patients with various tumors, particularly Lynch syndrome (Poulogiannis et al., 2010), which is a hereditary disease caused by a congenital dysfunction of DNA mismatch repair (MMR) system, referred to as mismatch repair deficiency (dMMR). Patients with this disease tend to develop tumors. Hence, it was suspected that MSI is related to carcinogenesis, regardless of tumor type. In a previous study, MSI was detected in 4/35 (11%) of dogs with mammary gland tumors (McNiel et al., 2007); but the MMR system was not analyzed in that study. Previously, it was determined that dMMR tended to occur in oral malignant melanoma and hepatocellular carcinoma in dogs (Inanaga et al., 2021). To the best of our knowledge, no reports have comprehensively analyzed MSI in various tumors of dogs and evaluated its relationship with dMMR.

Microsatellite instability mostly is induced by dMMR. The MMR system preserves genomic stability and is mainly regulated by 4 genes: *msh2*, *msh6*, *mlh1*, and

pms2 (Li, 2008). Mismatch repair deficiency is caused by a germline mutation (i.e., Lynch syndrome), somatic mutation, or hypermethylation of these MMR genes in various tumors (Fishel et al., 1993; Leach et al., 1993; Bronner et al., 1994; McGivern et al., 2004; Mensenkamp et al., 2014). It is known that dMMR increases tumor mutation burden and MSI, leading to carcinogenesis (Eshleman et al., 1995; Eshleman and Markowitz, 1996; Umar et al., 1997; Le et al., 2015). In humans, dMMR and MSI are simultaneously examined by immunohistochemistry (IHC) and PCR to understand genetic mechanisms in carcinogenesis (Richman, 2015). In addition, numerous reports showed that tumors with dMMR and MSI are highly responsive to immune checkpoint inhibitors (ICIs) (Diaz and Le, 2015; Kumar et al., 2017; Coventry et al., 2019). Therefore, dMMR/MSI serves as a predictive biomarker of the likely efficacy of ICIs (Yi et al., 2018). Furthermore, PCR for MS also provides information about loss of heterozygosity (LOH) and MSI. Loss of heterozygosity is another type of genomic instability and is caused by various mechanism such as somatic mutations, errors of meiosis, and mitotic recombination. It is known to be associated with tumors and developmental disorders (Engel, 1980; Eiriksdottir et al., 1995; O’Keefe et al., 2010). Thus, LOH is considered a prognostic biomarker in patients with tumors, independent of MSI (Grundeir et al., 2000).

I evaluated MSI status in several types of tumors to identify tumor types that

frequently have MSI, including its relationship with dMMR in clinical cases and cell lines. Based on these data, MS loci suitable for MSI analysis were narrowed down to make the analysis more productive and accurate. Furthermore, LOH was analyzed simultaneously with MSI because only few reports have examined LOH at MS loci in tumors of dogs.

MATERIALS & METHODS

Clinical samples

Clinical samples were obtained from tumor-bearing dogs that came to Yamaguchi University Animal Medical Center and Kyoto Animal Medical Center for diagnosis or treatment from May 2020 to March 2021. All dogs underwent biopsy or surgical resection and were diagnosed with malignant tumors by veterinary pathologists at Yamaguchi University or IDEXX Laboratories (Tokyo, Japan), except for some cases with lymphoma that were diagnosed only by cytology. Blood and fresh tumor tissues were collected from each dog to evaluate MSI, and an oral mucosal swab was taken instead of blood to extract normal tissue DNA when tumor cells were observed on a blood smear. Formalin-fixed paraffin-embedded (FFPE) tumor tissues were collected from each dog to evaluate dMMR. The study was approved by the Ethics Committee of Yamaguchi University.

PCR for microsatellite sequences

Genomic DNA was extracted from blood (or oral mucosal swab) and fresh tumor tissues using a QIAamp DNA Blood Mini Kit (QIAGEN Tokyo, Tokyo, Japan) or QIAamp DNA Mini Kit (QIAGEN Tokyo), respectively, and was stored at 4 °C until

further use.

For MSI analysis, 12 MS loci were selected (Table II-1) (Breen et al., 2001; Guyon et al., 2003) because of their high instability rates in a previous report (McNiel et al., 2007) and my preliminary experiments. Microsatellite sequences were amplified from tumor and normal DNA by PCR in 15 μ L of reaction volume, including 12 ng of DNA and 2.5 μ M of each primer labeled with fluorescent dye using BIOTAQ DNA Polymerase (Meridian Life Science, Inc., Memphis, TN). The PCR amplicons were detected using capillary electrophoresis (3500xL Genetic Analyzer, Thermo Fisher Scientific K.K., Tokyo, Japan) and analyzed using a software application (Peak Scanner, Thermo Fisher Scientific K.K.). In each MS sequence, electropherograms of tumor and normal DNA were compared and defined as MSI-positive if a difference in product size was detected. In addition, LOH was defined as a $\geq 35\%$ reduction in the peak of 1 allele in tumor DNA compared to that in normal DNA (Powierska-Czarny et al., 2003). The electropherograms of each sample were evaluated independently by 3 observers.

Immunohistochemistry for dMMR analysis

As described in a previous report (Inanaga et al., 2021), each FFPE tissue was incubated with mouse monoclonal anti-human MLH1 antibody (clone G168-15,

GeneTex, inc., California, U.S, diluted at 1:50), rabbit monoclonal anti-human MSH2 antibody (clone SP46, Abcam, Cambridge, U.K, diluted at 1:5100), or mouse monoclonal anti-human MSH6 antibody (clone 44/MSH6, BD Transduction Laboratories, California, U.S, diluted at 1:250). The isotype antibodies, rabbit IgG antibody (clone DA1E, Cell Signaling Technology, Inc., Massachusetts, U.S.) and mouse IgG1 antibody (clone P3.6.2.8.1, eBioscience, Inc., California, U.S), were used as negative immunostaining controls. A peroxidase stain DAB Kit (Nacalai Tesque, Inc., Kyoto, Japan) was used to visualize the immunoreaction. The sections were counterstained with Mayer's hematoxylin. To bleach the melanocytic granules of melanoma tissues, the sections were incubated in 10% hydrogen peroxide before antigen retrieval. A Simple Stain AEC Solution (Nichirei Bioscience Inc., Tokyo, Japan) was used to visualize the immunoreaction instead of DAB Kit.

Immunoreactivity was evaluated if the nuclei of the tumor cells were immunostained with each antibody as compared with those in isotype controls. The immunostaining of the normal tissue areas of each sample was used as a control and simultaneously evaluated to confirm positive immunostaining. Negative immunostaining was confirmed when the nuclei of tumor cells showed negative immunostaining. The immunoreactivity of each sample was independently evaluated by 4 observers. Based on

the results of IHC, the MMR status of each case was determined; MMR proficiency (pMMR) was confirmed if all 3 MMR proteins showed positive immunostaining, or dMMR was confirmed if ≥ 1 proteins showed negative immunostaining based on criteria used in studies of human tissues (Richman, 2015).

Cell culture

Human embryonic kidney (HEK) 293T cell line and SV40-immortalized canine gallbladder epithelial cells (kindly provided by Dr. Kenji Baba) were cultured in a Dulbecco's Modified Eagle Medium (DMEM)-based complete medium (DMEM mixed with 10% fetal bovine serum and 100 units/mL penicillin-streptomycin and 55 μ M 2-mercaptoethanol). All cell lines were maintained at 37°C in a humidified CO₂ gas incubator.

Establishment of dMMR/MSI cell lines

According to previous reports (Igase et al., 2019), CRISPR-Cas9 small guide RNAs (sgRNAs), which target 1 or more of the MMR genes (*mlh1*, *msh2*, and *msh6*), were designed using an online CRISPR design tool (<http://zlab.bio/guide-design-resources>). The sequences of sgRNAs of canine MLH1, canine MSH2, and canine MSH6

were 5'-GTGGTGAACCGCATCGCGGC, GCGCTTCTTCCAGGCCATGC-3', and 5'-GAAGTAAGGCCTAAGGTCCA-3', respectively. These oligonucleotides were synthesized and inserted into a lentiCRISPR v2 plasmid, which was donated by Dr. Feng Zhang (Addgene, Cambridge, MA, #52961; <http://n2t.net/addgene:52961>; RRID: Addgene 52961). To produce lentivirus-expressing sgRNAs that target each gene, HEK293T cells were seeded at 3.0×10^5 cells/well on a 6-well plate a day before transfection. On the day of transfection, 1.25 µg of each plasmid was mixed with PEI Max (Polysciences inc., Pennsylvania, U.S) in an OPTI-MEM buffer (Thermo Fisher Scientific K.K.), incubated for 15 min at room temperature, and added onto HEK293T cells. After 24 h, the supernatant of HEK293T cells was replaced with a DMEM-based complete medium without antibiotics. After another 24 h, the supernatant containing lentiviruses was collected and transferred onto gallbladder epithelial cells. After 12 h of infection, the supernatant was replaced with a DMEM-based complete medium, followed by the addition of 0.2 mg/mL hygromycin onto the gallbladder epithelial cells to select stably transduced cells. Individual single clones were isolated by limited dilution method and further analyzed by Western blotting to confirm the knockout of each protein. Based on the results of Western blotting, clones with complete knockout of each MMR gene were selected and passaged >30 times over 3 months after single clone isolation.

Western blotting analysis

Each cell line was collected and lysed with an NP-40 lysis buffer (1% NP40, 10 mM Tris HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA). Equal amounts of protein samples (30 µg) were electrophoresed in 7% acrylamide gel before transferring them onto membranes. The membranes were incubated with each primary antibody (same as the antibodies used in IHC) diluted at 1:1,000 in 0.5% skimmed milk in Tris Buffered Saline with Tween20 overnight at 4 °C. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (Merck Millipore, Darmstadt, Germany) or HRP-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch, Pennsylvania, U.S.) was used as a secondary antibody. The membranes were soaked in Western Lightning Plus-ECL (PerkinElmer, Tokyo, Japan) to visualize the immunoreaction. Immunoreactive bands were quantified and analyzed using AMERSHAM Image Quant 800 (Global Life Sciences Technologies Japan, Tokyo, Japan). The membranes were reused with mouse monoclonal anti-beta actin antibody (Sigma-Aldrich Japan K.K., Tokyo, Japan) to verify equal loading of proteins in each lane.

Immunocytochemistry

Canine gallbladder epithelial cells of the wild-type or with knockout of canine MMR genes *mlh1*, *msh2*, and *msh6* were cultured on microcover glasses (Matsunami Glass Kogyo, Osaka, Japan) in a 12-well culture plate. On the following day, cells were washed and fixed by 4% paraformaldehyde. Then, the cells were permeabilized by treatment with 0.1% Triton-X in phosphate buffered saline (PBS), and incubated in 5% bovine serum albumin and 5% skim milk in PBS to block non-specific protein activity. The cells were incubated with each antibody (same as the antibodies used in IHC), and followed by incubation with DyLight 488-conjugated goat anti-mouse IgG (Biolegend, San Diego, CA) or DyLight 488-conjugated goat anti-rabbit IgG (Abcam). The coverslips were mounted on glass slides with Molecular Probes Prolong Gold with DAPI (Invitrogen, Carlsbad, CA) for the staining of nuclei. Samples were visualized at BZ-X800 (KEYENCE, Osaka, Japan). The isotype antibodies were used as a negative immunostaining control.

Statistical analysis

Statistical analysis was performed using JMP Pro 15 software (JMP Japan, Tokyo, Japan). A Wilcoxon rank-sum test was conducted to determine the association between the number of MSI-positive markers and dMMR/pMMR status. Statistical

significance was set at $p < .05$.

A least absolute shrinkage and selection operator (LASSO) regression model and decision tree model were fitted to further select important variables for the association between dMMR/pMMR status (dependent variable) and MSI-positive markers (independent variables). The LASSO regression model shrank all regression coefficients toward zero and the coefficients of irrelevant variables to zero according to the regulation weight λ . The optimal value of λ was determined using a 5-fold cross validation. The decision tree analysis used the same variables as LASSO (dMMR/pMMR status as dependent variable and MSI-positive markers as independent variable). The tuning parameters were determined using a 5-fold cross validation. Ten cases with other tumors (Table 2) were excluded in the LASSO regression and the decision tree model analyses.

RESULTS

Study population

In this study, 101 dogs with tumors were included. The characteristics of these dogs are shown in Table II-2. The population consisted of 51 males (27 castrated) and 50 females (38 spayed). Median age at diagnosis was 11 years and 11 months (range, 5–16 years and 10 months). The cases were classified into 25 cases of oral malignant melanoma, 13 cases of lymphoma, 3 cases of mast cell tumor, 4 cases of mammary gland carcinoma, 5 cases of urothelial carcinoma, 9 cases of hepatocellular carcinoma, 9 cases of squamous cell carcinoma, 7 cases of adenocarcinoma, 3 cases of hemangiosarcoma, 11 cases of soft tissue sarcoma, and 12 cases of other tumors (Table II-3). Four lymphoma cases (cases 27, 29, 37, and 38 in Table II-2) and 1 urothelial carcinoma case (case 46 in Table II-2) had been treated before the tissues were collected for the study.

Microsatellite analysis for clinical cases

The results of MSI and LOH are shown in Table II-2. Microsatellite instability was defined as an alteration in the peak size of MS amplicons in tumor DNA compared to that in normal DNA (Figure II-1). At least 1 MSI-positive marker was detected in 20 cases of oral malignant melanoma, 5 cases of lymphoma, 4 cases of soft tissue sarcoma,

6 cases of hepatocellular carcinoma, 5 cases of squamous cell carcinoma, 3 cases of adenocarcinoma, 2 cases of urothelial carcinoma, 4 cases of mammary gland carcinoma, 2 cases of hemangiosarcoma, and 4 cases of others, whereas no MSI-positive marker was detected in mast cell tumors (Figure II-2). Oral malignant melanomas tended to have MSI markers more frequently.

Loss of heterozygosity was defined as a reduction in the peak of 1 allele in tumor DNA compared to that in normal DNA (Figure II-1) and was evaluated for all MS loci. In addition, some cases showed increased amplitude of the other allele, which could indicate copy neutral LOH (CN-LOH). Copy loss LOH (no difference in amplitude of the other allele) was observed frequently in markers located in chromosome of *Canis lupus familiaris* (CFA) 5 (FH3837, FH2594, and FH3113), CFA 16 (FH2175), and CFA 22 (C22.763) across tumor types (Figure II-3, Table II-2). Multiple MS markers on CFA 5 showed copy loss LOH in 4 cases (cases 22, 78, 89 and 93 in Table II-2)

Relationship between dMMR and MSI in clinical cases

The FFPE specimens of tumor tissues were obtained from 81 of 101 dogs. The canine MLH1, MSH2, and MSH6 (referred to cMLH1, cMSH2, and cMSH6) in these specimens were analyzed by IHC. The classifications of the 81 tumor specimens are

shown in Table II-4.

Representative results of IHC are shown in Figure II-4A and B. Based on criteria for dMMR used in humans, all analyzed cases were classified into pMMR or dMMR. The proportion of tumors with dMMR was 8/21 (38.1%) cases of oral malignant melanoma, 1/4 (25%) cases of mammary gland carcinoma, 1/5 (20%) cases of urothelial carcinoma, and 3/9 (33.3%) cases of hepatocellular carcinoma, whereas the remainder of the specimens were classified into pMMR (Table II-4, Table II-2). For 20 cases, including 2 dMMR cases (cases 1 and 46), out of 101 cases, FFPE samples of normal tissues also were available, and expression of MMR proteins was detected in all of them (Figure II-5).

Then, whether or not dMMR was associated with MSI in clinical cases was confirmed. Comparing the number of MSI-positive markers between cases with dMMR or pMMR in entire tumors, these markers were significantly higher in cases with dMMR (Figure II-4C, $p < .0001$). Further analysis of the relationship by tumor type identified significant results in oral malignant melanoma and hepatocellular carcinoma ($p < .01$ and $p < .02$, respectively) and entire tumors, but not in urothelial carcinoma and mammary gland carcinoma ($p = .11$ and $p = .08$, respectively). On the other hand, there was no relationship between dMMR and number of LOH (data not shown).

Statistical evaluation to select MS loci suitable for dMMR/pMMR status

To identify which MSIs contributed substantially to dMMR in tumors of dogs, the LASSO regression model and decision tree analysis were used. In LASSO, 6 variables were estimated as non-zero coefficients (but not statistically significant), which might indicate that the appearance of MSI in FH2305 and the total number of MSI-positive markers reflect dMMR (Table II-5).

Additionally, the decision tree model indicated that the cases with dMMR or pMMR could be classified with high probability by setting up nodes with ≥ 2 or ≤ 1 MSI-positive loci, respectively (Figure II-6).

Establishment of cell lines with dMMR and MSI analysis

I investigated whether dMMR induces MSI *in vitro* using canine cell lines after knockout of the MMR gene. A CRISPR/Cas9 system was used to establish the dMMR cell lines, and was followed by confirmation of knockout by Western blotting (Figure II-7) and immunocytochemistry (Figure II-8). No MLH1 protein was detected in both clones of the MLH1 knockout cell line. The MSH2 and MSH6 knockout cell lines expressed no MSH6 and MSH2, in addition to a defective expression of MSH2 and MSH6,

respectively, as shown in a previous study (Inanaga et al., 2021). The knocked-out cell lines and wild-type cells were serially passaged for 3 months to allow mutations to accumulate in MS loci. Then, the MSI status in these cell lines was analyzed at 2-time points (0 and 3 months). Based on results of the MSI analysis, an MSI-positive marker occurred in dMMR cell lines but not in wild-type cell lines from 0 to 3 months. At 3 months, 2 loci (FH2054 and AHT137) showed MSI in a cMLH1-deficient cell line, 2 loci (PEZ11 and FH3837) in a cMSH2-deficient cell line, and 4 loci (PEZ11, FH2054, FH2594 and AHT137) in a cMSH6-deficient cell line (Table II-6).

DISCUSSION

In this study, MSI and dMMR frequently were detected, mainly in canine oral malignant melanoma. A previous report using a different cohort indicated that oral malignant melanoma and hepatocellular carcinoma in dogs tended to have a defective MMR system (Inanaga et al., 2021), which is consistent with this current study. In addition, I found that dMMR was related to MSI in both clinical cases and cell lines, which was supported by the results in the LASSO regression and decision tree model. However, the number of MSI-positive markers in the cell lines and some clinical cases with dMMR was lower than expected based on a previous study of humans showing that knockout of MMR genes results in high frequencies of MSI (Mandal et al., 2019). There are several possible reasons for this difference: 1) presence of factors other than dMMR involved in the appearance of MSI; 2) other proteins that function as major MMR proteins; and 3) inappropriate MS loci used. Elevated MS alterations at selected tetra-nucleotide repeats (EMAST) especially must be considered because 8 of all 12 MS loci used in this study were tetra-nucleotide repeat sequences (Table II-1). The EMAST is caused by dysfunction of MSH3 (Haugen et al., 2008), which is 1 of the MMR proteins, rather than that of MSH2, MSH6, MLH1, and PMS2. It was reported that EMAST occurring at tetra-nucleotide MS consisted of 'AAAG' or 'ATAG'. In this study, 6 markers

(PEZ11, FH2305, PEZ8, FH2175, FH3837, FH2594) consisted of 'AAAG' repeats and 1 marker (FH2054) consisted of 'ATAG' repeats. However, the expression of MSH3 proteins was not examined in this current study.

In humans, MSI is evaluated by analysis of 5 selected MS loci, and a tumor sample is defined as MS stable (MSS) if all 5 sequences are normal, MSI-low (MSI-L) if 1 sequence is mutated, or MSI-high (MSI-H) if ≥ 2 sequences are mutated. High microsatellite instability is highly consistent with dMMR in various tumors (Cicek et al., 2011; Yamashita et al., 2018). Several studies reported that tumors with dMMR/MSI-H have approximately 25 times more mutations than other tumors (Le et al., 2015), suggesting that tumors with dMMR/MSI-H express a number of neoantigens and are more immunogenic compared to tumors with pMMR/MSS or pMMR/MSI-L. Thus, it was expected that tumors with dMMR/MSI-H might be sensitive to ICIs, particularly for PD-1 or PD-L1 inhibitors. In fact, pembrolizumab, a PD-1 inhibitor used in humans was reported in clinical trials to be effective in patients with dMMR/MSI-H solid malignancies that progressed after chemotherapy, regardless of tumor type (Le et al., 2017, 2020; Lemery et al., 2017; Marabelle et al., 2020). These results made pembrolizumab the first drug authorized for treatment based on MSI status rather than the pathological diagnosis (Marcus et al., 2019).

Recently, clinical trials of ICIs using canine PD-1 or PD-L1 inhibitors were reported in spontaneous tumors of dogs including oral malignant melanoma, and showed efficacy in some cases (Maekawa et al., 2017, 2021; Igase et al., 2020c). In these studies, some dogs with oral malignant melanoma experienced clinical benefits. Hence, in veterinary medicine, tumors with MSI/dMMR possibly could have a good response to PD-1 and PD-L1 inhibitors. Although few reports on ICI treatment for mammary gland carcinoma and hepatocellular carcinoma in dogs exist, studies evaluating its efficacy against these tumors are expected because of the high likelihood of MSI/dMMR.

As mentioned above, only 5 MS loci are used for MSI analysis in human medical practice. This MS panel is composed of mono- or di-nucleotide repeats because tri-nucleotide repeats are less altered than are mono- and di-nucleotide repeats (Dietmaier et al., 1997). Previous studies analyzed the MSI of multiple MS loci and MMR status and narrowed down the loci suitable for MSI analysis to 10 candidates (Bocker et al., 1997; Dietmaier et al., 1997). Based mainly on these reports, the National Cancer Institute recommended an MS panel for MSI analysis and defined the border between MSI-H and MSI-L in 1998 (Richard Boland et al., 1998). In this study, the appearance of MSI in C13.900, FH2054, CPH14, and AHT137 may particularly have relevance to dMMR however none of the results were statistically significant. Lack of significance was

probably a consequence of insufficient sample size. Moreover, our MS panel contained only a few di-nucleotide and no mononucleotide repeats. Furthermore, MSI analysis using mono- or di-nucleotide repeats is needed if the influence of EMAST also is considered. The border between MSI-H and MSI-L in tumors of dogs also should be decided.

Furthermore, LOH frequently was identified in the markers located in CFA 5, 16, and 22, suggesting mutations of tumor suppressor genes in the same region. Copy loss LOH in MS loci suggests that inactivation of nearby tumor suppressor genes may contribute to carcinogenesis (Eiriksdottir et al., 1995). In fact, tumor suppressor genes *TP53*, *WRN*, and *RBI* are located in CFA 5, 16, and 22, respectively (Guevara-Fujita et al., 1996). Thus, it was suspected that there were tumors with pMMR in this study caused by somatic deletion of the wild-type allele of these genes. Another study found that *TP53* is mutated in 16.7% of tumors in dogs (Alsaihati et al., 2021), which is similar to my results showing the detection of LOH at MS loci FH2594 in 15 cases. Tumor suppressor genes located in CFA 16 also may contribute to carcinogenesis. The CN-LOH was also detected sporadically across tumor types. Copy neutral loss of heterozygosity by mitotic recombination also can contribute to oncogenesis. For example, *BRCA1/2* and *Cyclin D1* are implicated in CN-LOH in tumors of humans (Walsh et al., 2008; Beà et al., 2009). In

dogs, oncogenes also may be involved in the occurrence of CN-LOH. However, I did not evaluate expression of such tumor suppressor genes and oncogenes in our study, and more detailed studies are needed.

In the present study, immunolabeling of PMS2 protein was not done because no monoclonal antibodies were certified to cross-react with canine PMS2 protein. In addition, the number of cases varied markedly depending on the tumor type. Because of the small number of cases of each tumor type, except for oral malignant melanoma, it will be necessary to recruit more tumor cases to evaluate the frequency of dMMR/MSI more accurately.

In conclusion, to my knowledge, this study is the first report in veterinary medicine to identify tumor types with frequent dMMR/MSI using a comprehensive analysis of dMMR and MSI. These results also suggesting a relationship between dMMR and MSI in clinical cases and *in vitro*. Additional studies are needed to consider other candidates.

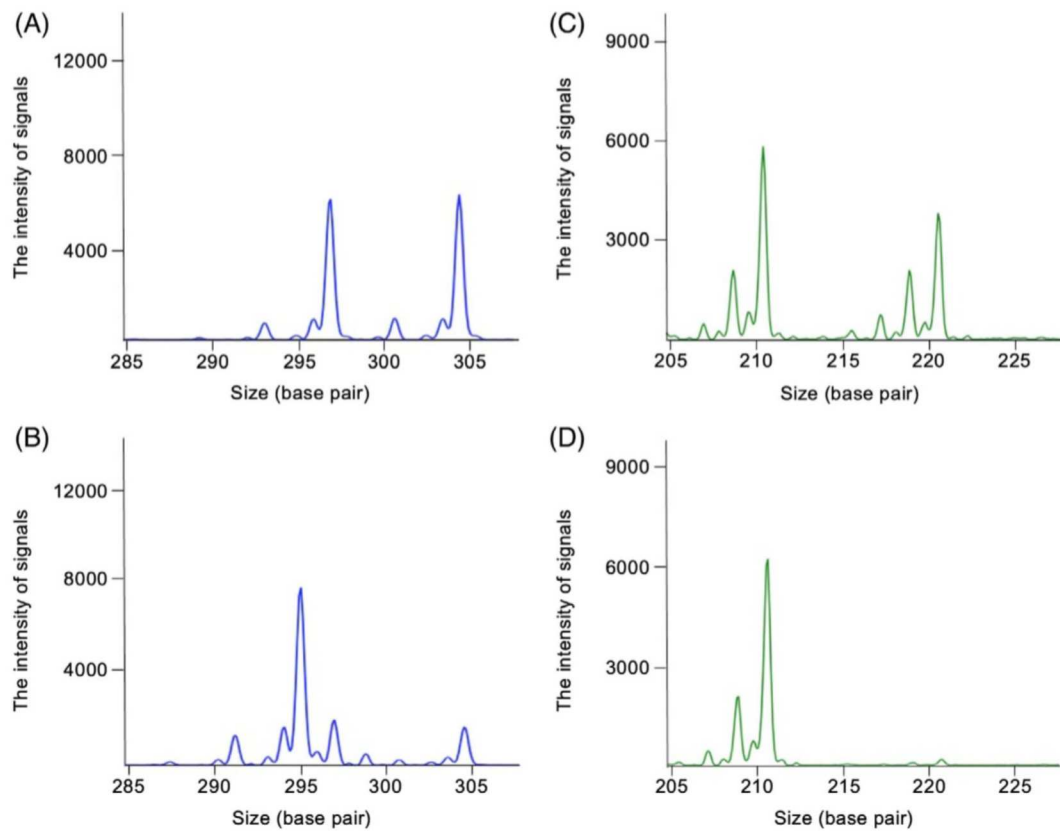


Figure II-1. Representative electropherogram of fluorescently tagged PCR amplicons of FH2594 (A and B) and FH3113 (C and D) from normal (A and C) and tumor (B and D) DNA. The intensity of fluorescent signal is shown on the y-axis and the size of the PCR amplicons (base pair) is shown on the x-axis. The alterations of peak size in B compared to A indicate microsatellite instability. The reduction of one allele in D compared to C indicates the loss of heterozygosity.

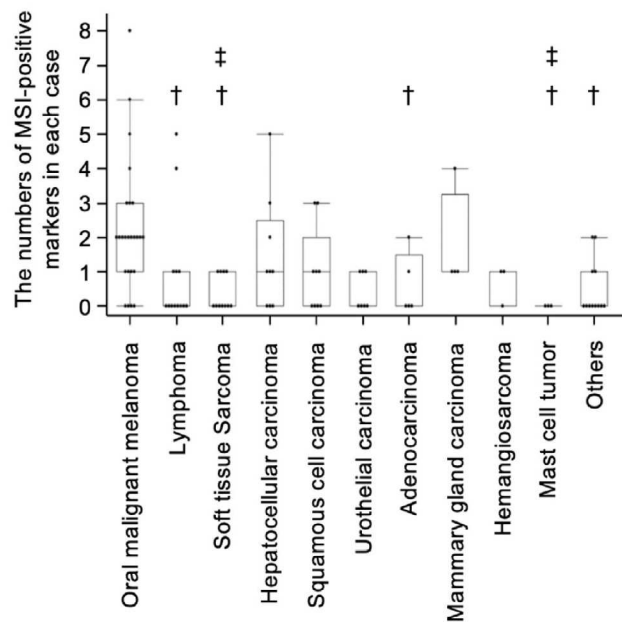


Figure II-2. Number of MSI-positive markers across the multiple type of canine tumors. All box-and-whisker plots represent the median (solid bars), interquartile range (boxes), and 1.5 x interquartile range (vertical lines). † and ‡ show significant differences vs oral malignant melanoma and mammary gland carcinoma, respectively (Wilcoxon rank-sum test).

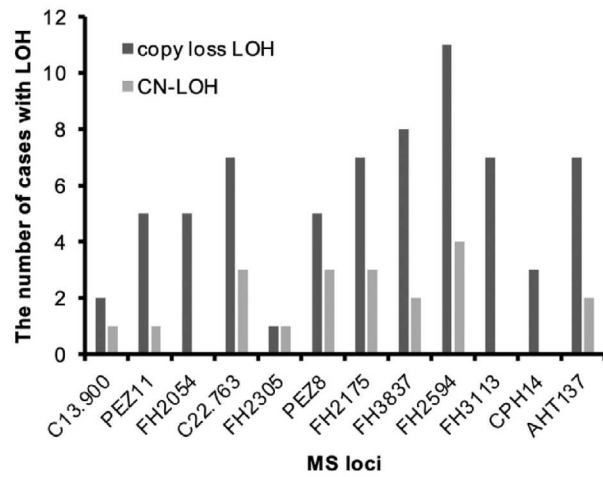


Figure II-3. Frequency of loss of heterozygosity (LOH) in canine tumors by microsatellite (MS) loci. The number of cases with LOH is shown on the y-axis, while the MS loci are shown on the x-axis. CN, copy neutral.

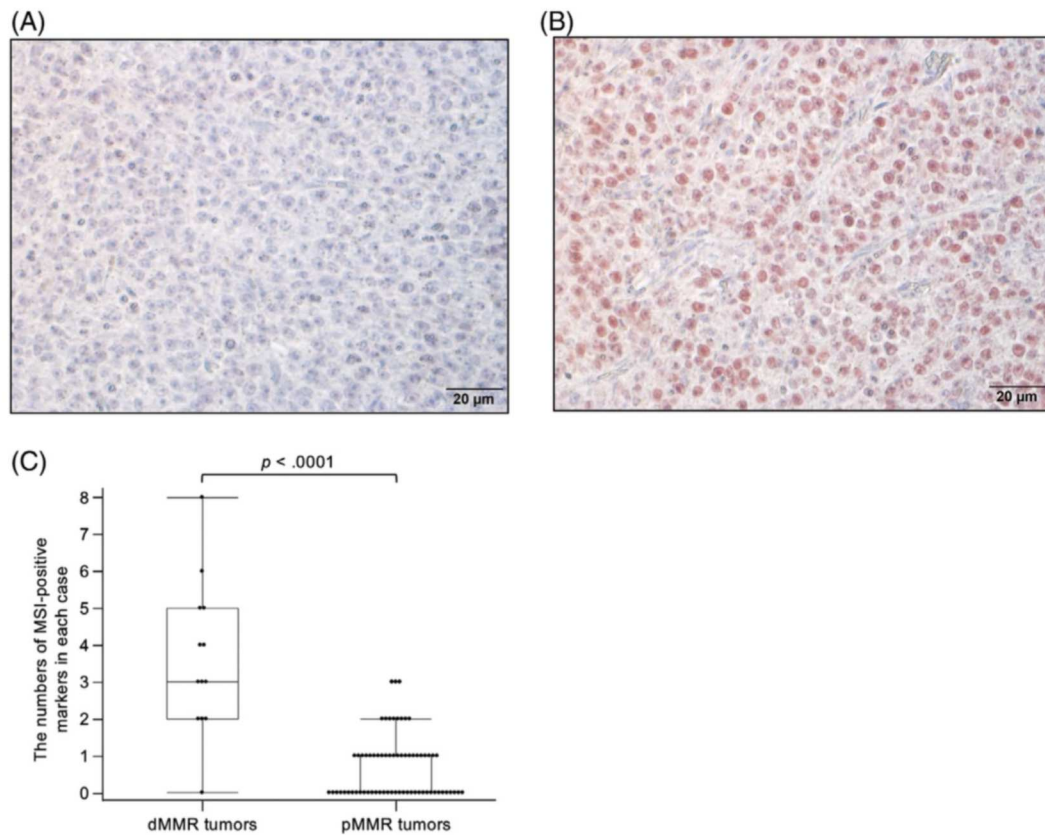


Figure II-4. Examples of immunohistochemistry demonstrating the loss of immunolabeling for cMLH1 (A) but retained immunolabeling for cMSH2 (B) in the nuclei of cells in a dog with oral malignant melanoma. These were counterstained with Mayer's hematoxylin. (C) Number of MSI-positive markers in each case by dMMR versus pMMR tumors ($p < .0001$, Wilcoxon rank-sum test). All box-and-whisker plots represent the median (solid bars), interquartile range (boxes), and 1.5 x interquartile range (vertical lines). MSI, microsatellite instability; dMMR, mismatch repair deficiency; pMMR, mismatch repair proficiency.

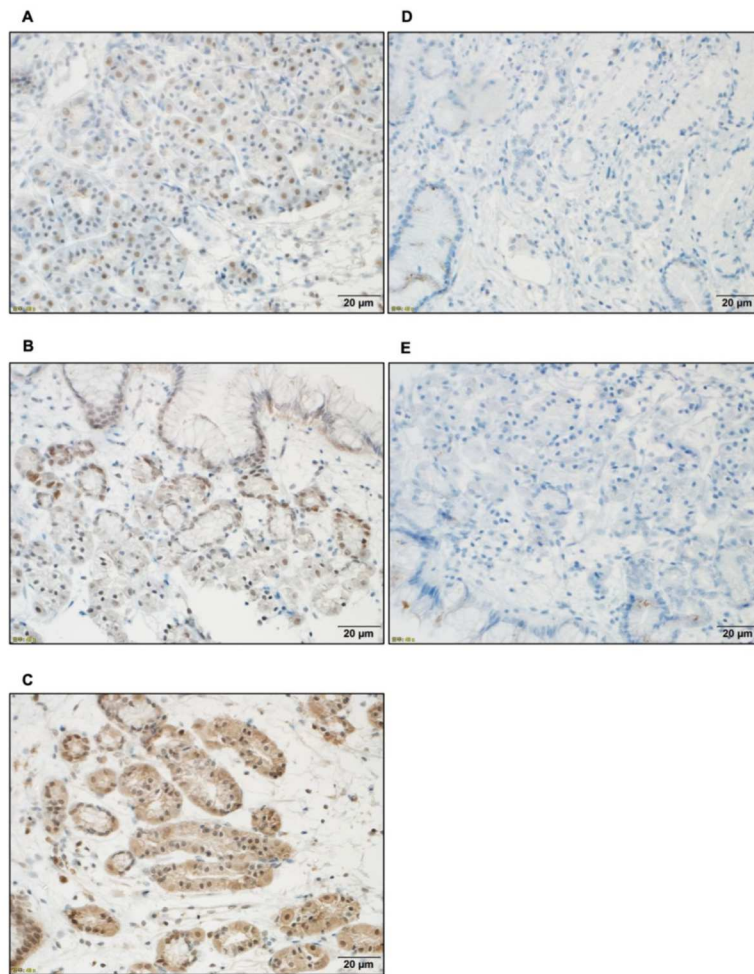


Figure II-5. Representative results of immunohistochemistry for canine MMR proteins in normal tissues from a case showing dMMR. This is case 46 with urothelial cancer showing negative immunostaining for MLH1, and positive immunostaining for MSH2 and MSH6, and this figure shows the immunostaining for cMLH1 (A), cMSH2 (B), cMSH6 (C), mouse IgG1 (D), and rabbit IgG, of normal duodenum in the same case.

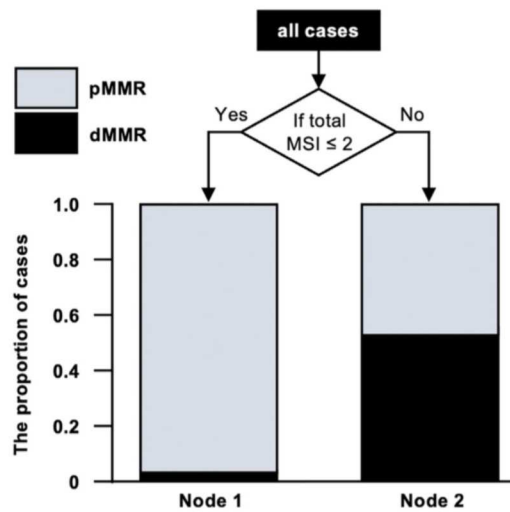


Figure II-6. Results of the statistical models performed to select the variables associated with dMMR. The decision tree model was used to identify the variants most associated with the MMR status. At node 1, 59 patients with ≤ 2 MSI-positive markers were classified as a group with a high probability of pMMR. At node 2, 12 patients with ≥ 3 MSI-positive markers were classified as a group with a high probability of dMMR. The graphs attached to the decision tree demonstrate the ratio of the cases with pMMR or dMMR at each node.

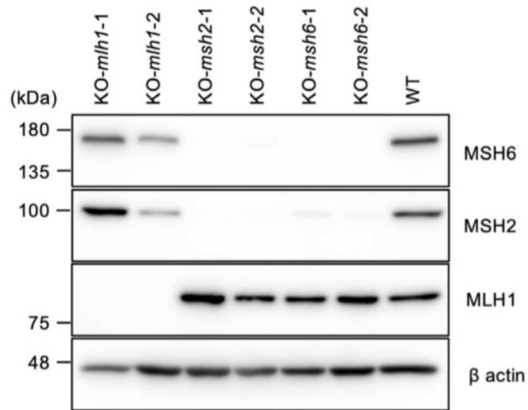


Figure II-7. All cell lysates were prepared from SV40-immortalized canine gallbladder epithelial cells of the wild-type or with knockout of canine mismatch repair genes *mlh1*, *msh2*, and *msh6*. Two of each gene-knockout cell line were cloned and evaluated for the knockdown of proteins. Proteins were separated by SDS-PAGE, followed by western blotting using anti-human MLH1 antibody, anti-human MSH2 antibody, or anti-human MSH6 antibody. β -actin was used as a protein loading control.

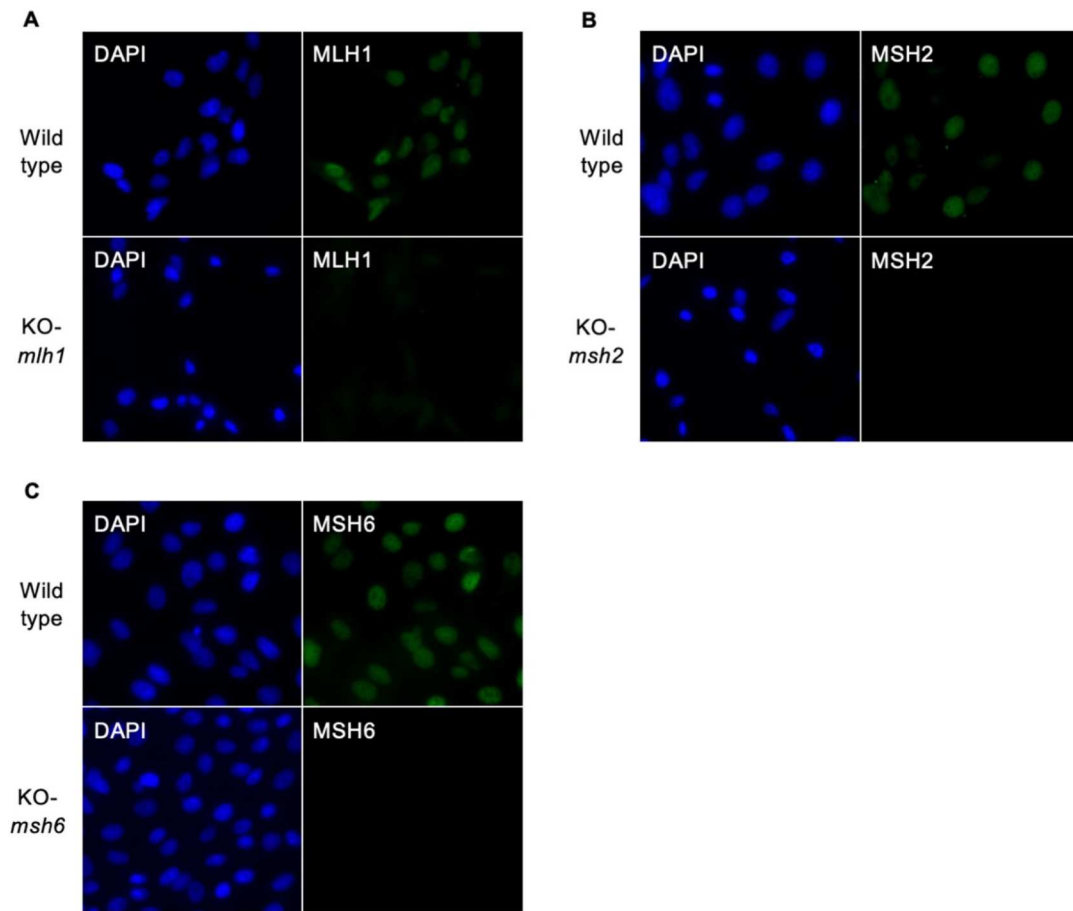


Figure II-8. Immunocytochemistry for canine MMR proteins in canine gallbladder epithelial cells of the wild-type or with knockout of canine MMR genes *mlh1*, *msh2*, and *msh6*. Immunolabeling for cMLH1 (A), cMSH2 (B), and cMSH6 (C).

Table II-1. Primers used for amplification of microsatellite markers in this study.

Microsatellite marker	Repeat type	Chromosome	Forward primer	Reverse primer	Fluorescent label
C13.900	Di	CFA 13	TTGGACTTCTAATTTTTCATT	CAACTGACTAAATCTCCTAATG	FAM
PEZ11	Tetra	CFA 8	ATTCTCTGCCTCTCCCTTTG	TGTGGATAATCTCTTCTGTC	FAM
FH2054	Tetra	CFA 12	GCCTTATTCATTGCAGTTAGGG	ATGCTGAGTTTGAACCTTCCC	PET
C22.763	Di	CFA 22	CAGCCCACTTCCTGGAAATA	GACCACTGTGCATTAAGCC	HEX
FH2305	Tetra	CFA 30	TCATTGTCTCCCTTTCCCAG	AAGCAGGACATTCATAGCAGTG	FAM
PEZ8	Tetra	CFA 17	TATCGACTTTATCACTGTGG	ATGGAGCCTCATGTCTCATC	PET
FH2175	Tetra	CFA 16	TTCATTGATTTCTCCATTGGC	AGGACTCTAAAACTTGCCTCC	HEX
FH3837	Tetra	CFA 5	GGCCTCGTAGAATACATTTGG	AGCAAGGAAGGCATCTGG	FAM
FH2594	Tetra	CFA 5	TTTAAGGAGCTGCTCATGCA	CTGAAATTCCTGGCCAGTA	FAM
FH3113	Tetra	CFA 5	CTGAATTATGGGAAACATGG	CAGGGAAGGAAGAAACAGC	HEX
CPH14	Di	CFA 5	GAAAGACAATCCCTGAAATGC	ACCCCATTTATGAGAATCATGT	FAM
AHT137	Di	CFA 11	TACAGAGCTCTTAACGGGTCC	CCTTGCAAAGTGTCATTGCT	PET

Table II-2. Characteristics and whole results of dogs included in this study.

Case No.	Case information					Results of microsatellite analysis*										Results of mismatch repair analysis*			
	Tumor type	Subtype	Breed	Sex**	Age	C13.90C	PEZ11	FH2054C22.76FH2305	PEZ8	FH2175FH3837	FH2594FH3111	CPH14AHT137	No. of MSI markers	MLH1	MSH2	MSH6	Status		
1	Oral malignant melanoma	Amelanotic	Beagle	M	10y0m					MSI	MSI	MSI	5	-	+	+	dMMR		
2	Oral malignant melanoma	Melanotic	Miniature Dachshund	SF	14y4m					MSI			2	+	+	+	pMMR		
3	Oral malignant melanoma	Melanotic	Miniature Dachshund	CM	15y10m					MSI		MSI	2	+	+	+	pMMR		
4	Oral malignant melanoma	Amelanotic	French Bulldog	SF	13y5m	MSI	MSI	MSI	MSI	CL-LOH	MSI	MSI	8	-	-	-	dMMR		
5	Oral malignant melanoma	Melanotic	Shiu Tzu	F	11y2m					CL-LOH			2	+	+	+	pMMR		
6	Oral malignant melanoma	Melanotic	Miniature Dachshund	CM	15y3m	MSI				MSI			2			NE			
7	Oral malignant melanoma	Melanotic	Beagle	CM	14y10m		CL-LOH		MSI	MSI		CL-LOH	2			NE			
8	Oral malignant melanoma	Melanotic	Miniature Dachshund	M	13y1m		MSI					CL-LOH	2	+	+	+	pMMR		
9	Oral malignant melanoma	Melanotic	Mixed breed	F	13y8m	CN-LOH				MSI		MSI	2			NE			
10	Oral malignant melanoma	Amelanotic	Beagle	F	11y2m			MSI				CL-LOH-CL-LOH	1	+	+	+	pMMR		
11	Oral malignant melanoma	Melanotic	Miniature Schnauzer	CM	7y4m	MSI		CL-LOH-CL-LOH	MSI	CL-LOH			3	-	+	+	dMMR		
12	Oral malignant melanoma	Amelanotic	Labrador Retriever	SF	15y4m							MSI	1			NE			
13	Oral malignant melanoma	Melanotic	Beagle	M	10y10m								0	+	-	-	dMMR		
14	Oral malignant melanoma	Amelanotic	English Cocker Spaniel	SF	14y7m			CN-LOH				CL-LOH-CN-LOH	1	+	+	+	pMMR		
15	Oral malignant melanoma	Amelanotic	Shiba	M	14y8m			MSI		CL-LOH			1	+	+	+	pMMR		
16	Oral malignant melanoma	Melanotic	Miniature Dachshund	SF	13y8m	MSI	MSI		MSI	CL-LOH			3	+	-	-	dMMR		
17	Oral malignant melanoma	Melanotic	Pug	CM	12y6m							MSI	2	+	-	-	dMMR		
18	Oral malignant melanoma	Amelanotic	Toy Poodle	SF	12y4m	MSI			MSI			MSI	4	+	+	+	dMMR		
19	Oral malignant melanoma	Amelanotic	Pug	SF	14y0m								0	+	+	+	pMMR		
20	Oral malignant melanoma	Amelanotic	Miniature Dachshund	M	13y7m					CN-LOH	MSI	CN-LOH	3	+	+	+	pMMR		
21	Oral malignant melanoma	Amelanotic	Kaninchen Dachshund	SF	11y4m	MSI					MSI		3	+	+	+	pMMR		
22	Oral malignant melanoma	Amelanotic	Toy Poodle	CM	11y2m	CL-LOH	CL-LOH			CL-LOH-CL-LOH-CL-LOH			0	+	+	+	pMMR		
23	Oral malignant melanoma	Melanotic	Miniature Dachshund	CM	16y10m			MSI	MSI				2	+	+	+	pMMR		
24	Oral malignant melanoma	Melanotic	Border Collie	M	9y0m							CL-LOH	2	+	+	+	pMMR		
25	Oral malignant melanoma	Amelanotic	Papillon	SF	14y11m	MSI	MSI	MSI	MSI	CN-LOH		CL-LOH	6	+	-	-	dMMR		
26	Lymphoma	B cell	French Bulldog	M	9y11m							CL-LOH-CL-LOH	0			NE			
27	Lymphoma	non T non B	Bulldog	M	5y11m	MSI							1			NE			
28	Lymphoma	T cell	Miniature Schnauzer	M	14y10m								0	+	+	+	pMMR		
29	Lymphoma	non T non B	Golden Retriever	M	10y2m					CL-LOH	CL-LOH		0			NE			
30	Lymphoma	B cell	Welsh Corgi	CM	5y0m								0			NE			
31	Lymphoma	B cell	Mixed breed	M	8y7m							MSI	1			NE			
32	Lymphoma	B cell	Cavalier King Charles Spaniel	SF	7y10m								0			NE			
33	Lymphoma	B cell	Miniature Dachshund	CM	14y11m							CL-LOH	0			NE			
34	Lymphoma	T cell	Pug	M	6y3m	MSI	MSI	MSI				MSI	5			NE			
35	Lymphoma	B cell	Beagle	F	9y10m								0			NE			
36	Lymphoma	B cell	Yorkshire Terrier	M	8y11m								0			NE			
37	Lymphoma	B cell	Miniature Dachshund	F	11y11m							CL-LOH	1			NE			
38	Lymphoma	T cell	West Highland White Terrier	SF	13y6m	MSI	CL-LOH-CN-LOH			MSI	MSI	MSI	4			NE			
39	Mast cell tumor		French Bulldog	M	10y9m								0			NE			
40	Mast cell tumor		Shiba	M	12y9m	CL-LOH-CN-LOH							0	+	+	+	pMMR		
41	Mast cell tumor		Jack Russell Terrier	SF	10y1m								0	+	+	+	pMMR		
42	Mammary gland carcinoma		Welsh Corgi	SF	12y5m							CL-LOH	1	+	+	+	pMMR		
43	Mammary gland carcinoma		Miniature Dachshund	SF	Unknown	MSI		MSI		CN-LOH-CN-LOH-CL-LOH	MSI	MSI	4	+	+	+	dMMR		
44	Mammary gland carcinoma		Mixed breed	SF	14y0m			MSI				LOH	1	+	+	+	pMMR		
45	Mammary gland carcinoma		Yorkshire Terrier	SF	10y11m							MSI	1	+	+	+	pMMR		
46	Urothelial carcinoma		Miniature Dachshund	CM	10y7m		CL-LOH-CL-LOH		MSI		CN-LOH	MSI	2	+	+	+	dMMR		
47	Urothelial carcinoma		Welsh Corgi	SF	11y11m				MSI				1	+	+	+	pMMR		
48	Urothelial carcinoma		Miniature Dachshund	CM	14y8m								0	+	+	+	pMMR		
49	Urothelial carcinoma		Shetland Sheepdog	F	9y5m	CL-LOH							0	+	+	+	pMMR		
50	Urothelial carcinoma		Shiu Tzu	SF	12y11m								0	+	+	+	pMMR		
51	Hepatocellular carcinoma	Moderately differentiated	Yorkshire Terrier	CM	7y2m						CN-LOH		CN-LOH	0	+	+	+	pMMR	
52	Hepatocellular carcinoma	Well-differentiated	Toy Poodle	SF	12y10m	MSI		MSI	MSI	CL-LOH	MSI	MSI	5	-	+	+	dMMR		
53	Hepatocellular carcinoma	Well-differentiated	Shiba	CM	13y6m						MSI	CN-LOH-CL-LOH	1	+	+	+	pMMR		
54	Hepatocellular carcinoma	Well-differentiated	Mixed breed	M	10y8m	MSI						MSI	2	-	-	-	dMMR		
55	Hepatocellular carcinoma	Well-differentiated	Miniature Schnauzer	CM	11y0m				MSI				1	+	+	+	pMMR		
56	Hepatocellular carcinoma	Well-differentiated	French Bulldog	CM	12y3m								0	+	+	+	pMMR		
57	Hepatocellular carcinoma	Poorly differentiated	Mixed breed	SF	13y0m				MSI		MSI		3	+	+	+	dMMR		
58	Hepatocellular carcinoma	Well-differentiated	Golden Retriever	SF	11y0m						MSI		1	+	+	+	pMMR		
59	Hepatocellular carcinoma	Well-differentiated	Mixed breed	SF	10y0m	CL-LOH-CL-LOH					CL-LOH	CN-LOH	0	+	+	+	pMMR		
60	Squamous cell carcinoma		Golden Retriever	M	9y5m								0	+	+	+	pMMR		
61	Squamous cell carcinoma		Chihuahua	F	7y5m								0	+	+	+	pMMR		
62	Squamous cell carcinoma		Chihuahua	CM	12y6m			MSI	MSI	MSI			3	+	+	+	pMMR		
63	Squamous cell carcinoma		Miniature Dachshund	CM	16y6m								0	+	+	+	pMMR		
64	Squamous cell carcinoma		Maltese	SF	9y4m								0	+	+	+	pMMR		
65	Squamous cell carcinoma		Toy Poodle	SF	12y7m				MSI			CL-LOH	1	+	+	+	pMMR		
66	Squamous cell carcinoma		Shiba	M	14y3m						MSI		1	+	+	+	pMMR		
67	Squamous cell carcinoma		Weimaraner	CM	7y2m	MSI			MSI		MSI	MSI	3	+	+	+	pMMR		
68	Squamous cell carcinoma		Miniature Schnauzer	M	13y6m							MSI	1	+	+	+	pMMR		
69	Adenocarcinoma	Lung adenocarcinoma	Toy Poodle	M	14y3m								0	+	+	+	pMMR		
70	Adenocarcinoma	Lung adenocarcinoma	Miniature Dachshund	SF	11y8m	CL-LOH				CN-LOH		CL-LOH	CL-LOH	0	+	+	+	pMMR	
71	Adenocarcinoma	Lung adenocarcinoma	Border Collie	SF	13y3m								0	+	+	+	pMMR		
72	Adenocarcinoma	Apocrine gland anal sac adenocarcinoma	Shiu Tzu	CM	10y0m							MSI	1	+	+	+	pMMR		
73	Adenocarcinoma	Apocrine gland anal sac adenocarcinoma	Chihuahua	SF	9y8m	MSI							1	+	+	+	pMMR		
74	Adenocarcinoma	Cervicofacial adenocarcinoma	Mixed breed	CM	10y9m							MSI	1	+	+	+	pMMR		
75	Adenocarcinoma	Thyroid adenocarcinoma	Golden Retriever	SF	8y1m								0	+	+	+	pMMR		
76	Hemangiosarcoma		Miniature Schnauzer	CM	12y10m					MSI	CL-LOH		1	+	+	+	pMMR		
77	Hemangiosarcoma		French Bulldog	CM	10y2m	MSI							1			NE			
78	Hemangiosarcoma		Miniature Dachshund	CM	10y4m					CL-LOH		CL-LOH-CL-LOH	0	+	+	+	pMMR		
79	Soft tissue sarcoma	Fibrosarcoma	Standard Poodle	SF	9y6m								0	+	+	+	pMMR		
80	Soft tissue sarcoma	Fibrosarcoma	Mixed breed	F	14y0m								0	+	+	+	pMMR		
81	Soft tissue sarcoma	Fibrosarcoma	Mixed breed	SF	8y10m					CN-LOH			0	+	+	+	pMMR		
82	Soft tissue sarcoma	Fibrosarcoma	Jack Russell Terrier	F	7y0m								0	+	+	+	pMMR		
83	Soft tissue sarcoma	Liposarcoma	Yorkshire Terrier	SF	14y9m			CN-LOH	MSI			CL-LOH	1	+	+	+	pMMR		
84	Soft tissue sarcoma	Undifferentiated pleomorphic sarcoma	Miniature Dachshund	SF	13y1m								1	+	+	+	pMMR		
85	Soft tissue sarcoma	Perivascular wall tumor	Toy Poodle	M	16y6m	MSI							1	+	+	+	pMMR		
86	Soft tissue sarcoma	Perivascular wall tumor	Shiu Tzu	CM	13y10m								0	+	+	+	pMMR		
87	Soft tissue sarcoma	Perivascular wall tumor	Siberian Husky	F	7y11m					MSI			1	+	+	+	pMMR		
88	Soft tissue sarcoma	Leiomyosarcoma	Miniature Dachshund	SF	15y6m							CL-LOH	1	+	+	+	pMMR		
89	Soft tissue sarcoma	Myosarcoma	Mixed breed	CM	11y11m					CL-LOH		CL-LOH-CL-LOH	0	+	+	+	pMMR		
90	Others	Osteosarcoma	Mixed breed	SF	9y7m								0			NE			
91	Others	Intranasal carcinoma	Shetland Sheepdog	CM	13y8m			MSI				CL-LOH	1	+	+	+	pMMR		
92	Others	Gastrointestinal stromal tumor	Toy Poodle	SF	9y7m	MSI		MSI				CL-LOH	CL-LOH	2	+	+	+	pMMR	
93	Others	Spleen interstitial tumor	Papillon	F	11y1m	MSI		MSI				CL-LOH-CL-LOH	CL-LOH	2	+	+	+	pMMR	
94	Others	Undifferentiated malignant tumor	Miniature Dachshund	F	11y6m								0	+	+	+	pMMR		
95	Others	Undifferentiated malignant tumor	Miniature Schnauzer	SF	11y10m				CL-LOH-CN-LOH		CN-LOH		0	+	+	+	pMMR		
96	Others	Chondrosarcoma	Papillon	F	15y6m								0	+	+	+	pMMR		
97	Others	Malignant non-epithelial tumor	Norfolk Terrier	SF	13y0m								0	+	+	+	pMMR		
98	Others	Malignant non-epithelial tumor																	

*MSI, microsatellite instability; LOH, loss of heterozygosity; CL-LOH, copy loss LOH; CN-LOH, copy neutral LOH; dMMR, mismatch repair deficiency; pMMR, mismatch repair proficient

**M, male; CM, castrated male; F, female; SF, spayed female

Table II-3. Classification of tumor tissues included in this study.

Tumor type	Number of cases
Oral malignant melanoma	25
Melanotic	18
Amelanotic	7
Lymphoma	13
T-cell	3
B-cell	8
non-T non-B	2
Mast cell tumor	3
Low grade	3
Mammary gland carcinoma	4
Simple carcinoma	4
Urothelial carcinoma	5
Urinary bladder to urethra	5
Hepatocellular carcinoma	9
Poorly differentiated	1
Moderately differentiated	1
Well-differentiated	7
Squamous cell carcinoma	9
Adenocarcinoma	7
Apocrine gland anal sac adenocarcinoma	2
Lung adenocarcinoma	3
Ceruminous adenocarcinoma	1
Thyroid adenocarcinoma	1
Hemangiosarcoma	3
Soft tissue sarcoma	11
Fibrosarcoma	4
Perivascular wall tumor	3
Leiomyosarcoma	1
Liposarcoma	1
Myxosarcoma	1
Undifferentiated pleomorphic sarcoma	1
Others	12
Osteosarcoma	1
Gastrointestinal stromal tumor	1
Spleen interstitial tumor	1
Histiocytic sarcoma	1
Carcinosarcoma	1
Undifferentiated malignant tumor	2
Intranasal carcinoma	1
Carcinoma nos	3
Sarcoma nos	2
Total	101

Table II-4. Mismatch repair status of tumor tissues in this study.

Tumor type	Total cases	dMMR cases (%)
Oral malignant melanoma	21	8 (38)
Lymphoma	1	0
Mast cell tumor	2	0
Mammary gland carcinoma	4	1 (25)
Urothelial carcinoma	5	1 (20)
Hepatocellular carcinoma	9	3 (33)
Squamous cell carcinoma	9	0
Adenocarcinoma	7	0
Hemangiosarcoma	2	0
Soft tissue sarcoma	11	0
Others	10	0

Abbreviation: dMMR, mismatch repair deficiency.

Table II-5. Summary of LASSO regression model used to identify the variants associated with mismatch repair deficiency.

Variants	Coefficients	Odds ratio	P-value	95% confidence interval
AHT137	1.21	3.34	.52	0.08-137.0
FH2305	1.27	3.57	.14	0.67-19.1
CPH14	0.87	2.38	.44	0.26-21.7
C13.900	1.26	3.52	.53	0.07-173.2
FH2175	−0.16	0.85	.86	0.14-5.35
Total MSI	0.33	14.2	.4	0.64-3.01

Table II-6. Results of microsatellite analysis in canine mismatch repair genes-knocked out cell lines.

	Microsatellite markers											
Cell lines	C13.900	PEZ11	FH2054	C22.763	FH2305	PEZ8	FH2175	FH3837	FH2594	FH3113	CPH14	AHT137
KO- <i>mlh1</i>			MSI									MSI
KO- <i>msh2</i>		MSI						MSI				
KO- <i>msh6</i>		MSI	MSI						MSI			MSI
Wild type							LOH					

Note: Blank cells indicates no alteration.

Abbreviations: LOH, loss of heterozygosity; MSI, microsatellite instability.

Chapter 3

Analysis of subcellular localization and its functional consequences
of canine KIT protein

SUMMARY

Activating mutations in the canine *c-kit* gene are major oncogenic drivers in mast cell tumors (MCTs) and gastrointestinal stromal tumors. Although aberrant KIT immunostaining patterns in MCTs—particularly diffuse cytoplasmic staining (pattern III)—are associated with poor prognosis, the molecular mechanisms underlying these patterns remain unclear. In this study, I investigated whether *c-kit* mutations affect KIT subcellular localization and the resulting biological consequences. Canine KIT mutants frequently observed in tumors, including I417_H421dup, S479I, N508I, and exon 11 mutations (W556_558delinsF and P572_N586dup), were expressed in Ba/F3 cell line—an IL-3-dependent murine pro-B cell line that survives only in the presence of IL-3—to assess cytokine-independent transformation. Exon 11 mutants induced cytokine-independent cell proliferation and constitutive phosphorylation of KIT and STAT5. Flow cytometry and immunocytochemistry revealed that exon 11 mutants showed markedly reduced membrane expression and predominantly localized to the endoplasmic reticulum (ER), whereas other mutants localized mainly to the plasma membrane or exhibited perimembranous aggregates. All KIT mutants showed partial accumulation in the Golgi apparatus. ER-retained exon 11 mutants maintained STAT5 phosphorylation even under tunicamycin-induced ER stress, and tunicamycin selectively induced cytotoxicity in all

non-exon 11 mutants tested. These findings indicate that ER localization enables exon 11 mutants to preserve survival signaling. Overall, these results demonstrate that *c-kit* exon 11 mutations induce abnormal ER localization of KIT, leading to STAT5-mediated proliferative and survival abilities. This mechanism provides a biological basis for the poor clinical outcomes associated with KIT immunostaining pattern III in canine tumors.

INTRODUCTION

Receptor tyrosine kinase KIT plays an essential role in cell proliferation and differentiation. Activating mutations in the *c-kit* gene, which encodes KIT protein, induce ligand-independent autophosphorylation and drive tumorigenesis in both humans and dogs. In canine tumors, *c-kit* mutations are common—occurring in approximately 30% of mast cell tumors (MCTs) and 35-70% of gastrointestinal stromal tumors (GISTs) (Gregory-Bryson et al., 2010; Takeuchi et al., 2013; Bonkobara, 2015; Takanosu et al., 2016; Morini et al., 2022). These mutations are associated with a higher histological grade and poor prognosis (Zemke et al., 2002; Webster et al., 2006).

In canine MCTs, immunohistochemistry (IHC) for KIT reveals distinct staining patterns among cases. Based on Kiupel's classification and subsequent studies (Kiupel et al., 2004; Gil da Costa et al., 2007), KIT expression can be categorized into three patterns: pattern I, membranous staining; pattern II, paranuclear focal staining; and pattern III, diffuse cytoplasmic staining. These staining patterns correlate with clinical behavior, with increasing risks of metastasis, recurrence, and shorter survival from pattern I to III (Kiupel et al., 2004; Thompson et al., 2011; Brocks et al., 2020). Such variation in KIT staining has been hypothesized to reflect aberrant subcellular localization caused by *c-kit* mutations, and numerous studies have investigated the relationship between KIT

localization abnormalities and *c-kit* mutations. However, the findings have been inconsistent across studies (Webster et al., 2006; Thompson et al., 2011; Chen et al., 2022), and a definitive causal relationship has not been established.

A similar issue has been described in canine GISTs, where cytoplasmic KIT staining is observed in a subset of cases but did not clearly correlate with underlying *c-kit* mutations (Takanosu et al., 2016). Likewise, a study examining canine oral malignant melanoma also failed to demonstrate a clear association between KIT staining and *c-kit* mutations (Chu et al., 2012).

In human mastocytosis and acute myeloid leukemia (AML), mutations within the tyrosine kinase domain of KIT are well characterized. In particular, approximately 30% of patients with core-binding factor AML harbor the D816V mutation, which is associated with poor prognosis (Cairolì et al., 2005). In addition, gain-of-function mutations in exon 11 such as $\Delta 560-578$ and K642E are detected in nearly 80% of cases in human GISTs, and also associated with poor prognosis (Gupta et al., 1998; Quek et al., 2016). Furthermore, aberrant subcellular localization of mutant RTKs has been well reported in human cancers, where such mislocalization can activate signaling pathways distinct from those of wild-type proteins (Jaramillo et al., 2012; Song et al., 2025).

By extension, it is reasonable to hypothesize that mislocalization associated with mutation also occurs in canine KIT. however, this has yet to be conclusively demonstrated. This gap in knowledge may be explained by the fact that most prior studies rely on IHC analysis of clinical samples, which is limited in its ability to resolve subcellular localization at single-cell resolution. Furthermore, molecular studies directly evaluating the functional consequences of *c-kit* mutations *in vitro* are lacking.

To address these limitations, the present study transduced canine *c-kit* mutations into cultured cells and aimed to characterize the subcellular localization of each KIT mutants at single cell resolution, and to determine how subcellular localization affects downstream signaling, cell proliferation, and cell survival. Through this approach, I sought to elucidate the molecular mechanisms underlying the association between intracellular KIT staining patterns and poor prognosis observed in canine tumors.

MATERIALS & METHODS

Cell culture

The retroviral packaging cell line Platinum-GP (Plat-GP) was kindly provided by Dr. Toshio Kitamura (The University of Tokyo, Tokyo, Japan). The murine pro-B cell line Ba/F3 (RCB1464) and the human embryonic kidney HEK293T (RCB2202) were obtained from the RIKEN BioResource Research Center (RIKEN BRC, Tsukuba, Japan). Plat-GP and HEK293T cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (D10 complete medium). Ba/F3 was maintained in RPMI1640 medium supplemented with 10% FBS and 10 ng/ml of mouse interleukin-3 (mIL-3; Peprotech, Cranbury, NJ). All cell lines were maintained at 37°C in a humidified 5% CO₂ gas incubator.

Reagent

Imatinib was obtained from Cell Signaling Technology, Inc. (Danvers, MA). Tunicamycin was obtained from Sigma-Aldrich Japan K.K. (Tokyo, Japan). Chlorpromazine was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Monensin was obtained from Thermo Fisher Scientific, K.K. (Tokyo, Japan). Stock solutions of the above reagents were formulated by dimethyl sulfoxide (DMSO; Sigma-Aldrich Japan

K.K., Tokyo, Japan). All reagent were stored at -20°C .

Construction of mutant *c-kit* vectors and retroviral expression vectors

The retroviral vectors expressing canine wild type and N508I mutant of *c-kit*, pMXs-KITWT-IRES-GFP and pMXs-KITN508I-IRES-GFP, were obtained from Dr. Akane Tanaka (Tokyo University of Agriculture and Technology, Tokyo, Japan) (Amagai et al., 2015). In these vectors, EGFP is co-expressed with *c-kit* via an internal ribosome entry site (IRES), allowing simultaneous expression of *c-kit* and EGFP in transduced cells. In addition, to obtain the other mutant forms of canine *c-kit*, PCR-based molecular techniques were used to transduce the desired mutations. All primer sequences used in this study are listed in Table III-1. Additional mutant forms of canine *c-kit* were generated as follows.

The pMx-KIT-ex8ITD-IG#1 plasmid was constructed by amplifying two fragments of *pMXs-KITWT-IRES-GFP* using primer sets YTM178/YTM2263 and YTM2264/YTM179, followed by overlapping PCR with primers YTM178/YTM179. The resulting PCR product was digested with BamHI and NotI and inserted into the BamHI- and NotI-digested *pMXs-KITWT-IRES-GFP* backbone.

The pMx-KIT-S479I-IG#1 construct was generated in the same manner using

primer sets YTM178/YTM2816 and YTM2815/YTM179, followed by overlapping PCR with YTM178/YTM179, BamHI-NotI digestion, and ligation into the *pMXs-KITWT-IRES-GFP* vector.

The pMx-KIT Δ 556_558-IG#8 plasmid was produced by first replacing the NcoI-SalI fragment of *pMXs-KITWT-IRES-GFP* with that of *pMXs-IP* to create *pMX-KITWT-IP*. Two fragments of *pMXs-KITWT-IRES-GFP* were then amplified using primer sets YTM2794/YTM2261 and YTM2262/YTM2795, joined by overlapping PCR with YTM2794/YTM2795, and inserted into the EcoRI- and NotI-digested *pMX-KITWT-IP* vector using the In-Fusion cloning system (Takara Bio, Shiga, Japan).

The pMx-KIT-ex11ITD-IG#1 construct was generated by overlapping PCR using fragments amplified with primer sets YTM178/YTM2814 and YTM2812/YTM179, followed by BamHI-NotI digestion and subcloning into the *pMXs-KITWT-IRES-GFP* vector. All constructs were verified by Sanger sequencing and next-generation sequencing (NGS) performed by Azenta Life Sciences (South Plainfield, NJ, USA) to confirm the presence of the intended mutations and the absence of unwanted sequence alterations.

Establishment of Ba/F3 cell lines overexpressing mutant canine c-kit

Ba/F3 cell line was retrovirally transduced with mutant canine *c-kit* vectors. Briefly, Plat-GP cell lines were transfected with one of the *c-kit* expression vectors and pCAGGS-VSVG using Lipofectamin®3000 (Thermo Fisher Scientific, K.K.). After 48h, the supernatant was collected and added to Ba/F3 cell line for infection. Finally, *c-kit* transduced Ba/F3 cell lines were selected by GFP expression using cell sorter (SONY Corp., Tokyo, Japan).

Cell proliferation assay

For the cell proliferation assay, each Ba/F3 cell line was seeded in triplicate in 96 well plates at 5.0×10^3 cells/well with or without 10 ng/ml of mIL-3 or 10 ng/ml canine stem cell factor (cSCF; R&D Systems, Minneapolis, MN), and cultured for 48h. After 48h of incubation, Cell Counting Kit-8 (CCK8; DOJINDO, Kumamoto, Japan) was added to each well, and cells were cultured for an additional 2h. Following this 2h incubation, the absorbance at 450 nm was measured in the plates using the VARIOSKAN LUX (Thermo Fisher Scientific K.K.). The same experiments were repeated three times.

Flow cytometry analysis

To confirm KIT expression on the plasma membrane, the cells were washed with

flow cytometry buffer (phosphate buffered saline with 2% FBS and 0.1% NaN₃), and transferred into 96-well U bottom plate at 2.0×10^5 cells/well, followed by incubation with PE-conjugated anti-KIT antibody (Table III-2) and rabbit IgG isotype control (eBioscience, San Diego, CA). After incubation at 4°C for 30 min, the cells were washed and then stained with propidium iodide at room temperature for 10 min, followed by flow cytometric analysis using Attune NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific K.K.) with FlowJo software (BD Biosciences, San Jose, CA).

Western blotting analysis

To evaluate the activation status of the mutant *c-kit*, each cell line was seeded in 6 well plates at 5.0×10^5 cells/well and incubated for 24h in the plain RPMI. After incubation, cells were stimulated with cSCF (10 ng/ml) for 5 min and harvested and lysed with NP40 lysis buffer (1% NP40, 10 mM Tris HCl (pH 7.5), 150 mM NaCl, and 1 mM EDTA). The protein lysates were electrophoresed in 8% acrylamide gel before transferring onto membranes. The membranes were incubated with each primary antibody (Table III-2) overnight at 4°C. HRP-conjugated anti-mouse IgG antibody (Merck Millipore, Darmstadt, Germany) or HRP-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch, Philadelphia, PA) was used as a secondary antibody. The membranes

were soaked in Chemi-Lumi One Super (Nacalai Tesque, Inc.) to visualize the immunoreaction. Immunoreactive bands were quantified and analyzed with iBright1500 (Thermo Fisher Scientific K.K.). The membranes were reprobed with mouse monoclonal anti-beta actin antibody (Sigma-Aldrich Japan K.K.) to verify equal loading of proteins in each lane.

Immunohistochemistry

According the previous report (Inanaga et al., 2021), I conducted immunohistochemistry in formalin-fixed and paraffin-embedded samples of canine cutaneous MCTs collected from several pathological laboratories (Yamaguchi University and Veterinary Pathology Diagnostic Center, Fukuoka, Japan). Briefly, tissues were incubated in 3% hydrogen peroxide for 30 min at room temperature (RT), followed by incubating in 5% skimmed milk for 30 min at RT. Next, tissues were incubated overnight at 4°C with anti-KIT antibody (Table III-2), before incubating for 30 min at RT with Histofine Simple Stain rabbit MAX PO (Nichirei Bioscience Inc., Tokyo, Japan). Then, Peroxidase Stain DAB Kit (Nacalai Tesque, Inc.) was used to visualize the immunoreaction. All stained samples were classified three KIT immunostaining patterns according to the previous report: pattern I consisted of tumor cells stained primarily along

the plasma membrane with only minimal cytoplasmic staining, pattern II was characterized by tumor cells with focally clustered cytoplasmic staining or a strong positive stippling throughout the cytoplasm, and pattern III was characterized by tumor cells with diffuse cytoplasmic staining (Kiupel et al., 2004).

Immunocytochemistry

According the previous report (Inanaga et al., 2024), I conducted immunocytochemistry using the antibodies (Table III-2). For detection of protein disulfide isomerase (PDI), cells were subsequently incubated with Alexa 555-conjugated goat anti-rabbit IgG (Cell Signaling Technology, Inc.). Samples were visualized at BZ-X800 All-in-One fluorescence microscope (KEYENCE, Osaka, Japan). Each image showed single sections with a 100× oil immersion objective, adjusted to give the same x, y, and z position in the all channels. The built-in optical sectioning mode was used to reject out-of-focus light.

Cytotoxicity assay

For the cell cytotoxicity assay, each Ba/F3 cell line was seeded in triplicate in 96 well plates at 5.0×10^3 cells/well in the presence of 1 µg/ml of tunicamycin, and cultured

for 48h. After 48h of incubation, cell viability was counted by trypan blue exclusion test according the previous report (Inanaga et al., 2024).

Statistical analysis

The ANOVA, Tukey–Kramer test, and Dunnett’s test determined the significance of differences among samples. These was performed using JMP Pro 15 software (SAS Institute Japan, Tokyo, Japan). Statistical significance was set at $p < 0.05$.

RESULTS

Association of KIT immunostaining patterns with histological grade in canine cutaneous mast cell tumors

First, I performed IHC for KIT protein in cases with canine cutaneous MCTs (Figure III-1A) to confirm whether the KIT immunostaining could be classified into three distinct patterns (I, II, and III). Consistent with previous report (Kiupel et al., 2004), three patterns of KIT immunostaining were observed in this study as well.

Next, I examined the association between KIT immunostaining patterns and histological grade in canine cutaneous MCTs. The distribution of KIT patterns according to histological grade was as follows: pattern I consisted of 22 low-grade cases, KIT pattern II consisted of 3 low-grade and 3 high-grade cases, and KIT pattern III consisted of 1 low-grade and 3 high-grade cases (Figure III-1B). These findings indicate that histological malignancy tends to increase from pattern I to III, suggesting that the subcellular localization of KIT is associated with histopathological malignancy of tumor cells.

Establishment of canine KIT mutants in Ba/F3 cells

To elucidate the mechanism of KIT subcellular localization, I established Ba/F3

cells expressing several canine *c-kit* mutants (Figure III-2) that have been frequently reported in canine tumors (Letard et al., 2008). I then examined the cell proliferation of each Ba/F3 cell in the presence or absence of mIL-3 and cSCF (Figure III-3A). All Ba/F3 cells expressing mutated KIT exhibited cytokine-independent cell proliferation. Particularly, the cells expressing exon 11 mutants (W556_558delinsF and P572_N586dup) showed significantly stronger proliferative ability than other mutants (Figure III-3A, $p < 0.05$). In contrast, Ba/F3 cells expressing wild-type KIT (KIT-WT) proliferated only in the presence of cytokines, IL-3 and SCF. The KIT mutants were phosphorylated even in the absence of cSCF as shown by western blotting (Figure III-3B), whereas KIT-WT was not phosphorylated in the absence of cSCF. Furthermore, the exon 11 mutants exhibited the immature forms of KIT (~110 kDa) compared with KIT-WT, and these immature forms were also phosphorylated (Figure III-3C). In addition, the exon 11 mutants showed a tendency of higher ratio of phosphorylated KIT relative to total KIT expressing than the other mutants (Figure III-3C).

Because each Ba/F3 cell line overexpressed the corresponding KIT protein, I considered the possibility that differences in expression levels might affect the proliferative activity among the mutants. The retroviral expression vectors used in this study contained an IRES sequence, allowing bicistronic expression of EGFP together

with KIT. Western blotting analysis revealed no apparent differences in EGFP expression levels among the mutants, and the KIT/EGFP ratio was also comparable (Figure III-3C). Therefore, I concluded that the effect of expression level on cell proliferation was negligible in this system.

In addition, I investigated the activation of KIT downstream signaling pathway in each Ba/F3 cell line by western blotting (Figure III-3B and S1). Interestingly, phosphorylation of STAT5 was observed only in cells expressing exon 11 mutants (Figure III-3B), whereas phosphorylations of other signaling proteins such as STAT3, Akt, and Erk were not differed specific to exon 11 mutants (Figure III-S1).

Subcellular localization of mutant KIT proteins

To confirm whether *c-kit* mutations affect the subcellular localization of KIT, I evaluated the expression of KIT on the plasma membrane by flow cytometry (Figure III-4A). The levels of KIT expression on the plasma membrane varied among the mutants. KIT-N508I was expressed on the membrane at levels comparable to those of KIT-WT, whereas KIT-I417_H421dup and KIT-S479I showed lower membrane expression than KIT-WT. The exon 11 mutants exhibited the most marked reduction in membrane expression compared with KIT-WT.

Next, I examined the KIT subcellular co-localization with organelles by immunocytochemistry to identify the KIT localization sites. KIT-WT and KIT-N508I were predominantly localized on the plasma membrane, which was consistent with the flow cytometry (Figure III-4B). KIT-I417_H421dup and -S479I formed aggregates beneath the plasma membrane, showing a perimembranous aggregated pattern. In contrast, the exon 11 mutants were mainly localized to the endoplasmic reticulum (ER). All KIT variants, including the WT, showed partial localization to the Golgi apparatus (Figure III-4C). These findings suggest that distinct *c-kit* mutations differentially affect the intracellular trafficking and membrane localization of KIT.

c-kit exon 11 mutants maintain the downstream signals for cell survival in ER

The exon 11 mutants induced phosphorylation of STAT5 and were localized to the ER (Figures III-3B and III-4B). These findings led me to hypothesize that ER localization of these mutants might contribute to their enhanced cell survival and proliferation. To test this hypothesis, each Ba/F3 cell line was treated with tunicamycin. Tunicamycin induces accumulation of KIT in the ER by inhibiting *N*-linked glycosylation during glycoprotein synthesis. Ba/F3 cells expressing exon 11 mutants significantly maintained cell proliferation and survival, whereas cells expressing other KIT variants

were induced cell death by tunicamycin treatment (Figure III-5A and 5B, $p < 0.05$).

Next, I performed western blotting analysis in Ba/F3 cells expressing KIT variants after treatment with tunicamycin. The molecular weight of each KIT protein was reduced in all Ba/F3 cell lines due to inhibition of *N*-linked glycosylation. Nevertheless, exon 11 mutants were able to activate STAT5 even in presence of tunicamycin (Figure III-5C, $p < 0.05$). In contrast, STAT5 was not phosphorylated in the cells expressing other KIT mutants. In addition, the phosphorylations of KIT and STAT5 were not observed in Ba/F3 cells expressing KIT-WT (Figure III-5D). Thus, it is suggested that phosphorylation of STAT5 associated with ER retention is a signaling pathway specific to exon 11 mutants.

Previous studies in human have shown that KIT mutants are degraded slower than KIT-WT due to their constitutive kinase activities (Honegger et al., 1987; Obata et al., 2014), suggests KIT mutants circulate among intracellular organelles. Based on this interpretation, I hypothesized that inhibition of other organelles trafficking would cause exon 11 mutants to accumulate in organelles other than the ER, thereby reducing STAT5 phosphorylation in cells expressing these mutants. To test this hypothesis, Ba/F3 cells were treated with monensin and chlorpromazine (CPZ), which inhibit transport from the Golgi apparatus and clathrin-mediated endocytosis, respectively. Treatment with either

inhibitor resulted in reduced cell proliferation across all KIT mutants, including exon 11 mutants (Figure III-6, $p < 0.05$). These findings suggest that ER retention of exon 11 mutants is essential for sustaining downstream proliferative signaling.

Sensitivity to tyrosine kinase inhibitor in Ba/F3 cells expressing KIT variants

To investigate the association between *c-kit* mutations and sensitivity to tyrosine kinase inhibitor, each Ba/F3 cell was treated with imatinib for 48h (Figure III-7, $p < 0.05$). Ba/F3 cells expressing KIT-mutants were sensitive to imatinib compared to KIT-WT. In particular, the cells expressing exon 11 mutants exhibited most sensitive to imatinib among the KIT mutants.

DISCUSSION

This study demonstrated that the *c-kit* exon 11 mutants exhibited aberrant localization to the ER, where they enhance STAT5 phosphorylation and thereby confer stronger proliferative and survival abilities than other KIT mutants (Figure III-8). To the best of my knowledge, no previous studies have investigated aberrant subcellular localization of RTKs in canine tumors at the molecular level. Thus, the present study is the first to elucidate this mechanism in dogs.

In human tumors, several mutant receptor tyrosine kinases (RTKs) are also known to acquire abnormal subcellular localization (Tabone-Eglinger et al., 2008; Bougherara et al., 2009; Song et al., 2025). For instance, FLT3-ITD, KIT-N822K, KIT-D816V accumulates in the ER, Golgi apparatus, and endosome, respectively, driving signaling pathways distinct from the WT proteins (Choudhary et al., 2009; Obata et al., 2014, 2019; Lv et al., 2021). The ER localization and STAT5 activation observed in the canine *c-kit* exon 11 mutants resemble these human aberrantly localized RTKs, suggesting that, in addition to ligand-independent autophosphorylation, abnormal subcellular localization may contribute to the acquisition of malignant phenotypes in canine RTKs. Conversely, a previous human study demonstrated that exon 11 mutants ($\Delta 560-578$ and K642E) localized to the Golgi apparatus and activated STAT5 signaling (Quek et al.,

2016). This subcellular localization differs from our findings. Regarding this discrepancy, two possible explanations can be considered. First, the mutation sites are different between the human exon 11 mutants examined in that study and the canine mutants, which may affect their localization. Second, the molecular mechanisms regulating the intracellular trafficking of KIT may inherently differ between humans and dogs.

In the canine exon 11 mutants, plasma membrane expression was reduced whereas ER localization was markedly increased. Because the ER is broadly distributed throughout the cytoplasm, the ER-localization appeared as diffuse cytoplasmic staining in immunofluorescence, which was consistent with the pattern III staining observed in IHC. Furthermore, all KIT mutants, including WT, exhibited the part of accumulation at Golgi apparatus, likely due to overexpression, resembling the IHC pattern II. These findings suggest that pattern III reflects ER localization specific to exon 11 mutants, and pattern II may correspond to increased KIT expression levels. This interpretation is consistent with previous reports that both pattern II and III are associated with poor prognosis (Freytag et al., 2021). However, the relationship between KIT expression levels and accumulation at Golgi apparatus was not fully evaluated in this study and requires further investigation.

In human KIT, aberrant localization patterns have also been reported in other

mutants (Shi et al., 2016). Among them, the N505I mutant exhibited plasma membrane expression, which is consistent with my finding that canine KIT-N508I maintained membrane localization. However, orthologous mutations corresponding to canine KIT mutants other than N508I are not frequent in humans and have not been well characterized (Youk et al., 2016). Therefore, not only the ER localization seen in exon 11 mutants but also the aberrant localization tendencies of exon 8 ITD and S479I—partially suggested in this study—may serve as useful model for rare human KIT mutants.

In addition, CPZ has been reported to exert anti-tumor effects in human tumors, especially acute myeloid leukemia, by disrupting intracellular trafficking of aberrantly localized RTKs (Rai et al., 2020; Fujii-Hanamoto et al., 2025). CPZ inhibited cell proliferation regardless of the mutant type in this study, suggesting that targeting RTK localization may be a viable therapeutic strategy in canine tumors. However, it is remained unclear whether the CPZ-induced anti-tumor effect is directly attributable to altered KIT localization, and the underlying mechanism require further elucidation.

I also demonstrated that exon 11 mutants exhibited higher sensitivity to imatinib than other mutants. To the best of my knowledge, no previous studies have directly compared imatinib sensitivity among multiple canine KIT mutants. Our findings raise the possibility that the cases with IHC pattern III may be responsive to imatinib.

Conversely, clinical studies using toceranib and sunitinib have reported limited efficacy in the cases with pattern III (Weishaar et al., 2017). Therefore, large-scale prospective clinical studies are warranted to appropriately integrate IHC patterns, *c-kit* mutations, and drug sensitivities into therapeutic decision. Moreover, point mutations on tyrosine kinase domain of KIT contribute to the acquisition of resistance to tyrosine kinase inhibitors (Bosbach et al., 2012; Shi et al., 2016; Nakano et al., 2017). Therefore, it is necessary to examine the entire coding region of the *c-kit* gene for potential mutations.

This study has several limitations. First, exon11 contains diverse deletion and ITD patterns, but I focused only on representative high-frequency variants; thus, the influence of other variants on localization and signaling remains unknown. Second, although the present data indicate that ER localization enhances STAT5 activation, the detailed molecular mechanisms remain to be clarified. Further investigation into this pathway will be necessary to establish aberrant ER localization as a therapeutic target.

In conclusion, the c-kit exon11 mutants aberrantly localize to the ER and enhance STAT5 phosphorylation, contributing to the acquisition of malignant phenotypes. This phenomenon corresponds to the pattern III staining observed in IHC and highlights the pathological and clinical significance of RTK subcellular localization in canine tumors.

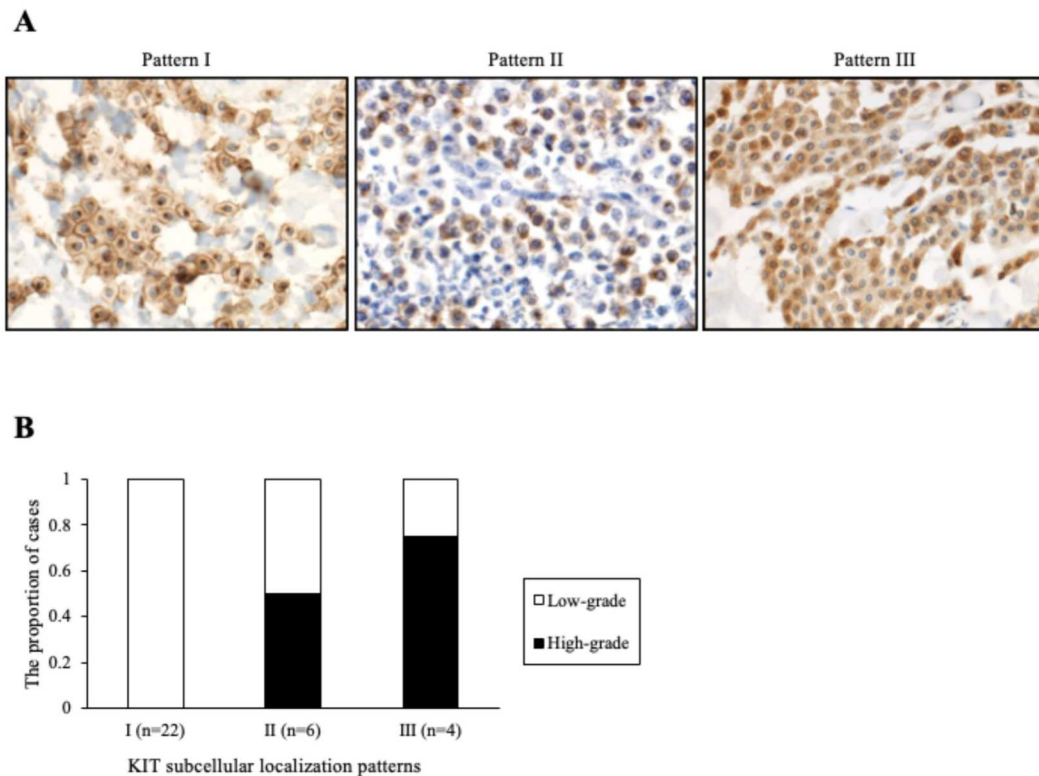


Figure III-1. KIT immunostaining patterns were associated with histological grade.

(A) Representative results of immunohistochemistry demonstrating each KIT immunostaining pattern in the dogs with cutaneous mast cell tumors. These were counterstained with Mayer's hematoxylin. Pattern I shows plasma membrane staining. Pattern II shows focal or stippled cytoplasmic staining. Pattern III shows diffuse cytoplasmic staining. (B) The proportions of cases with low- or high-grade canine cutaneous mast cell tumor in each KIT immunostaining pattern.

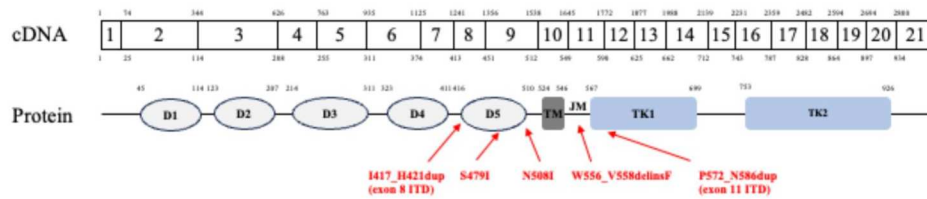


Figure III-2. Schematic representation of the structure of canine *c-kit* gene and KIT protein.

Boxes show the exon structure of canine *c-kit* cDNA, and the numbers in each box indicate the exon number. The number above and below each box indicates the numbers of the first nucleotide and amino acid in each exon, respectively. Protein structure is shown below the DNA structure. Numbers below the protein structure indicate the amino acid numbers of each domain. D1-D5 refer to immunoglobulin domain. Each arrow indicates a mutation used in this study. TM, transmembrane domain; JM, juxta-membrane domain; TK, tyrosine kinase domain.

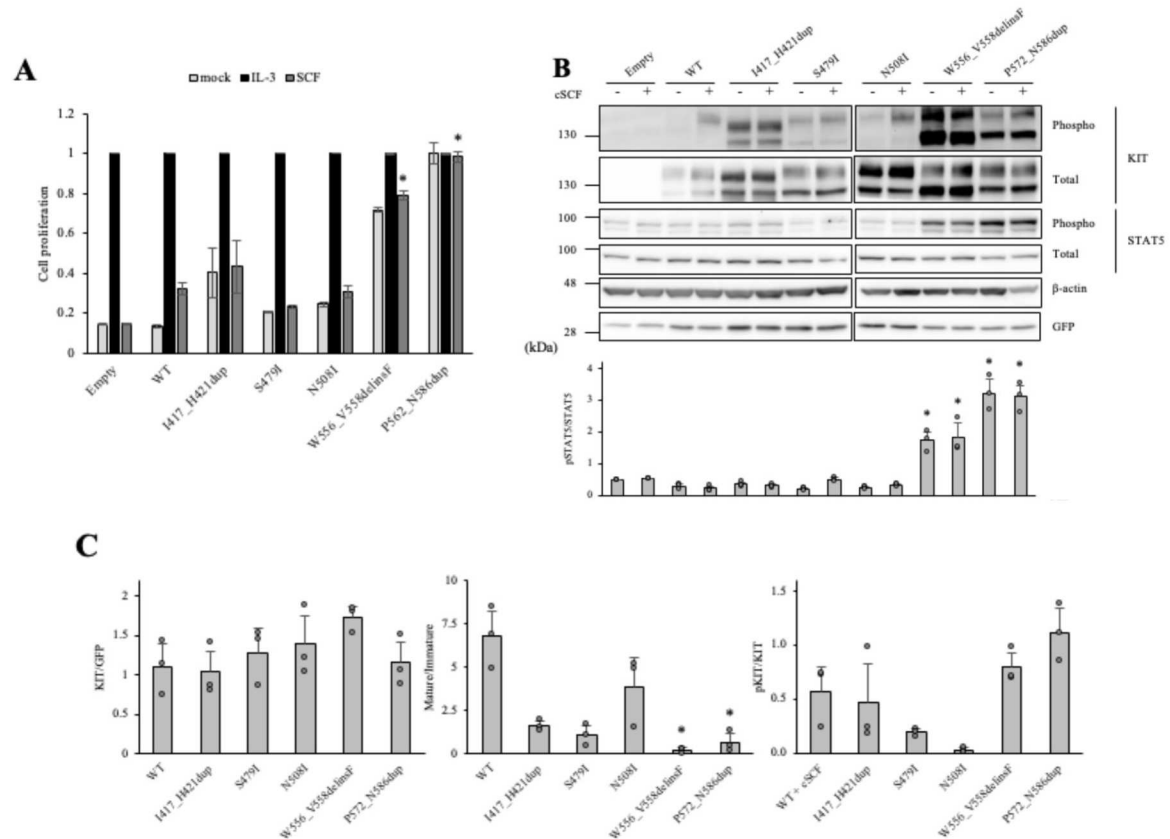


Figure III-3. Canine *c-kit* mutations on exon 11 up-regulate the cell proliferation and STAT5 phosphorylation compared to other mutants in Ba/F3 cells.

(A) Cell proliferation assay in Ba/F3 cells expressing each *c-kit* mutant. Cells were treated with 10 ng/ml of mIL-3, 10 ng/ml of cSCF, or mock for 48 hours, and cell proliferation rate was counted by Cell Counting Kit-8 reagent. The y-axis indicates the proliferation ratio at cells at each treatment when mIL-3-treated cells were set as 1. The results were expressed as mean $\pm$ SD of three independent experiments. Asterisks shows $p < 0.05$ vs other mutants. Tukey-Kramer test. mIL-3, mouse interleukin-3; cSCF, canine stem cell

factor. (B) The phosphorylation of KIT and STAT5 were analyzed by western blotting. β -actin was used as a loading control. Each blot represents one of three experiments showing a similar trend. The graphs show the expression levels of each protein quantified using ImageJ software. Data represent mean $\pm$ SD of three independent experiments. Tukey-Kramer test. *, $P < 0.05$ vs other mutants except for exon 11 mutants. (C) The graphs show the expression ratios (KIT/GFP, mature type/immature type of KIT, and pKIT/KIT). Each protein quantified using ImageJ software. Data represent mean $\pm$ SD of three independent experiments. Tukey-Kramer test. *, $P < 0.05$ vs KIT-WT and -N508I.

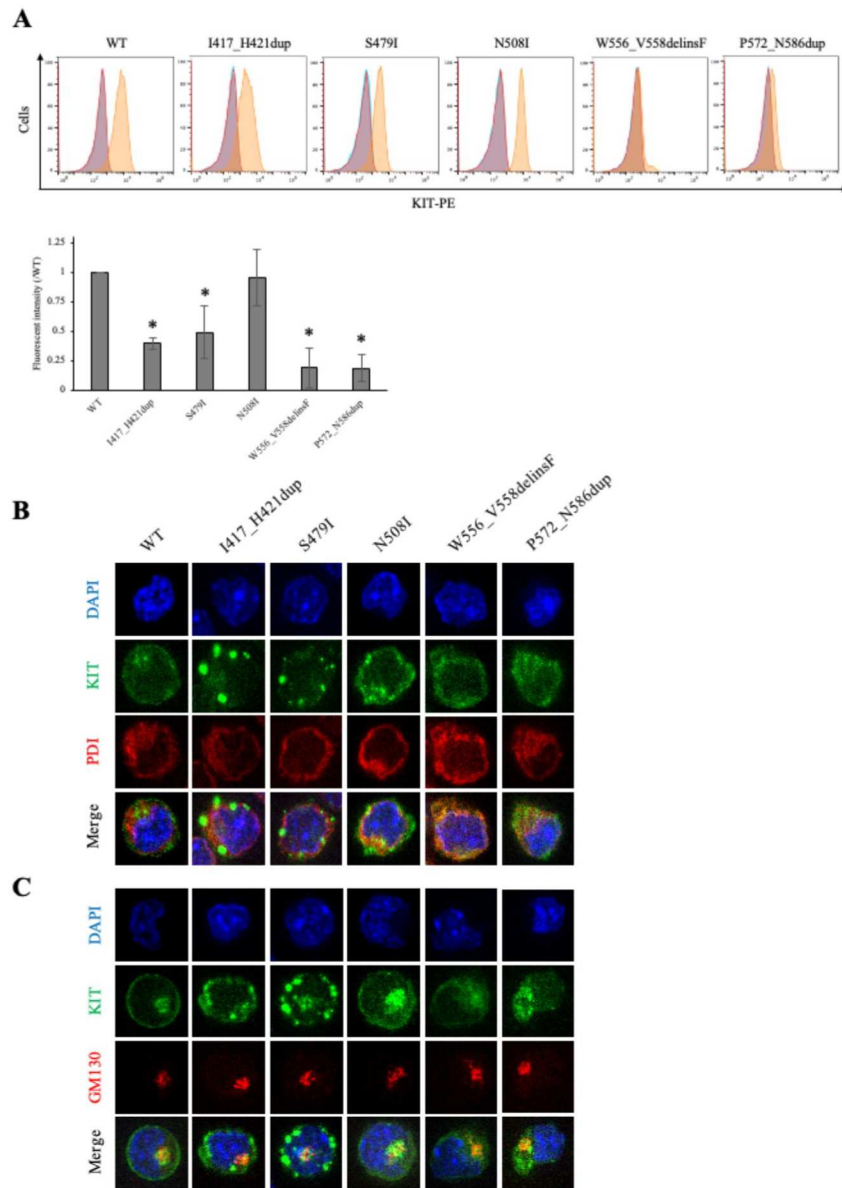


Figure III-4. *c-kit* mutations on exon 11 induce subcellular localization of KIT protein in Ba/F3 cells.

(A) KIT mutants expressed on the plasma membrane of Ba/F3 cells were stained with phycoerythrin (PE)-conjugated anti-KIT antibodies, and cell membrane-bound antibodies

were detected using flow cytometry analysis. The level of each KIT expression on the cell membrane (y-axis) was presented as the ratio of KIT-WT. The results were expressed as mean $\pm$ SD of three independent experiments. Asterisks shows $p < 0.05$ vs WT. Dunnett's test. (B) Each Ba/F3 cell was co-immunostained for KIT (green), PDI (red), and DAPI (blue). (C) Each Ba/F3 cell was co-immunostained for KIT (green), GM130 (red), and DAPI (blue).

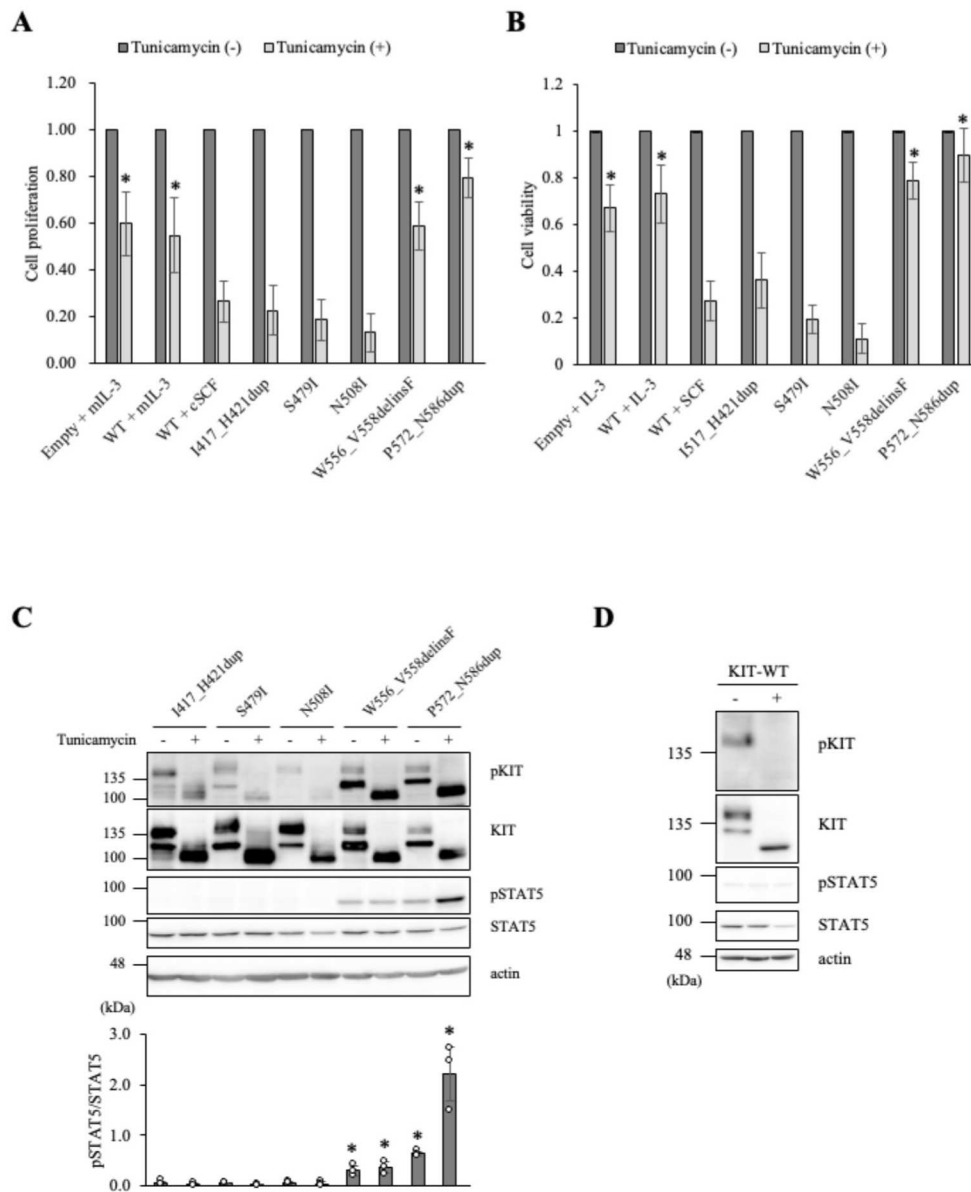


Figure III-5. Ba/F3 cells expressing *c-kit* exon 11 mutants activate cell proliferation signals in ER.

(A) Cell proliferation assay in each Ba/F3 cell treated with 1 μ g/ml of tunicamycin for 24h. Cell proliferation rate was counted by Cell Counting Kit-8 reagent. The y-axis

indicates the proliferation ratio at cells at each treatment when vehicle-treated cells were set as 1. The results were expressed as mean $\pm$ SD of three independent experiments. Asterisks shows $p < 0.05$ vs WT + cSCF, I417_H421dup, S479I, and N508I. Tukey-Kramer test. mIL-3, mouse interleukin-3; cSCF, canine stem cell factor. (B) Cytotoxicity assay in each Ba/F3 cell treated with 1 μ g/ml of tunicamycin and for 48h. Cell viability was counted by trypan blue exclusion test. The y-axis indicates the cell viability at cells at each treatment. The results were expressed as mean $\pm$ SD of three independent experiments. Asterisks shows $p < 0.05$ vs WT + cSCF, I417_H421dup, S479I, and N508I. Tukey-Kramer test. mIL-3, mouse interleukin-3; cSCF, canine stem cell factor. (C) The phosphorylation of KIT and downstream signals were analyzed by western blotting. Cells were treated with 1 μ g/ml of tunicamycin and no cytokines for 24h. β -actin was used as a loading control. Each blot represents one of three experiments showing a similar trend. The graphs show the expression levels of each protein quantified using ImageJ software. Data represent mean $\pm$ SD of three independent experiments. Tukey-Kramer test. *, $P < 0.05$. (D) The phosphorylation of KIT-WT and downstream signals were analyzed by western blotting. Cells were treated with 1 μ g/ml of tunicamycin and 10 ng/ml of cSCF for 24h. β -actin was used as a loading control.

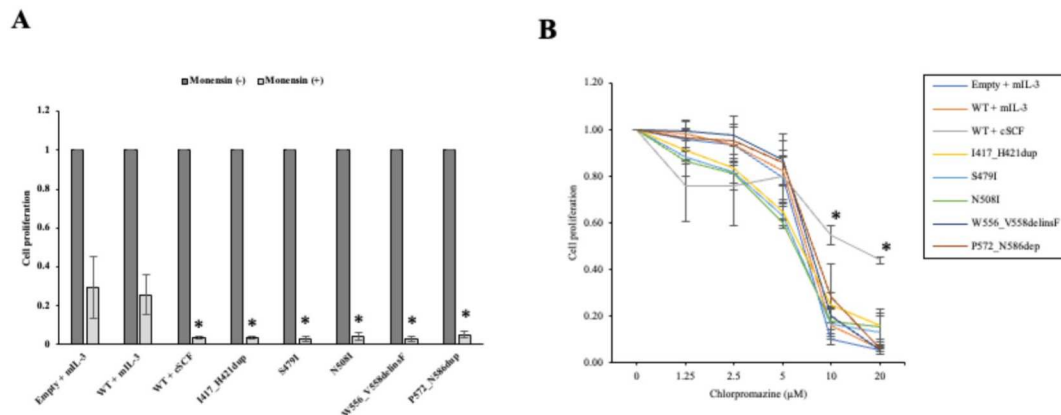


Figure III-6. Ba/F3 cells expressing *c-kit* exon 11 mutants depend on ER retention for cell proliferation.

(A) Cell proliferation assay in each Ba/F3 cell treated with 200 nM of monensin for 48h. Cell proliferation rate was counted by Cell Counting Kit-8 reagent. The y-axis indicates the proliferation ratio at cells at each treatment when vehicle-treated cells were set as 1. The results were expressed as mean $\pm$ SD of three independent experiments. Asterisks shows $p < 0.05$ vs Empty + mL-3 and WT + mL-3. Tukey-Kramer test. mL-3, mouse interleukin-3; cSCF, canine stem cell factor. (B) Cell proliferation assay in each Ba/F3 cell treated with chlorpromazine at the indicated concentration for 48h. Cell proliferation rate was counted by Cell Counting Kit-8 reagent. The y-axis indicates the proliferation ratio of cells at each treatment when milliQ-treated cells were set as 1. The results were expressed as mean $\pm$ SD of three independent experiments. Asterisks shows $p < 0.05$ vs other cells. Tukey-Kramer test.

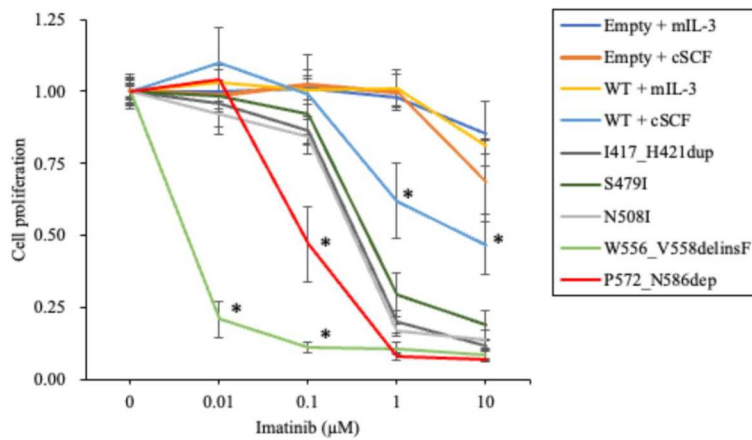


Figure III-7. Ba/F3 cells expressing *c-kit* exon 11 mutants were sensitive to imatinib.

Cell proliferation assay in each Ba/F3 cell treated with imatinib at the indicated concentration for 48h. Cell proliferation rate was counted by Cell Counting Kit-8 reagent.

The y-axis indicates the proliferation ratio of cells at each treatment when DMSO-treated cells were set as 1. The results were expressed as mean $\pm$ SD of three independent experiments. Asterisks shows $p < 0.05$ vs other cells. Tukey-Kramer test.

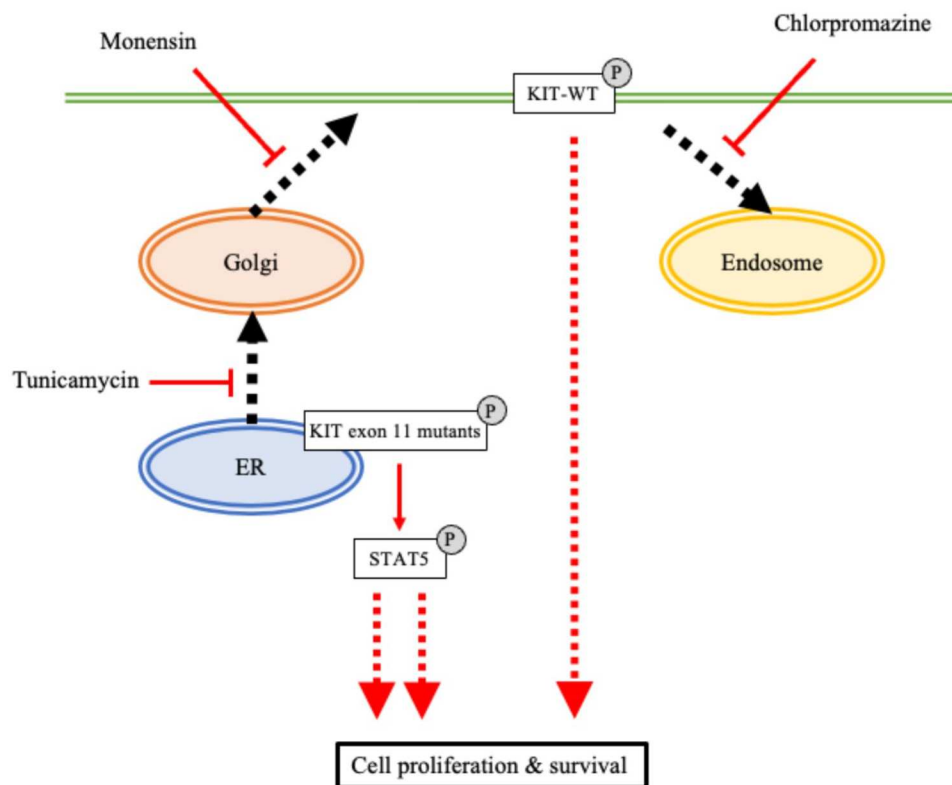


Figure III-8. Summary of intracellular trafficking of KIT-exon 11 mutants.

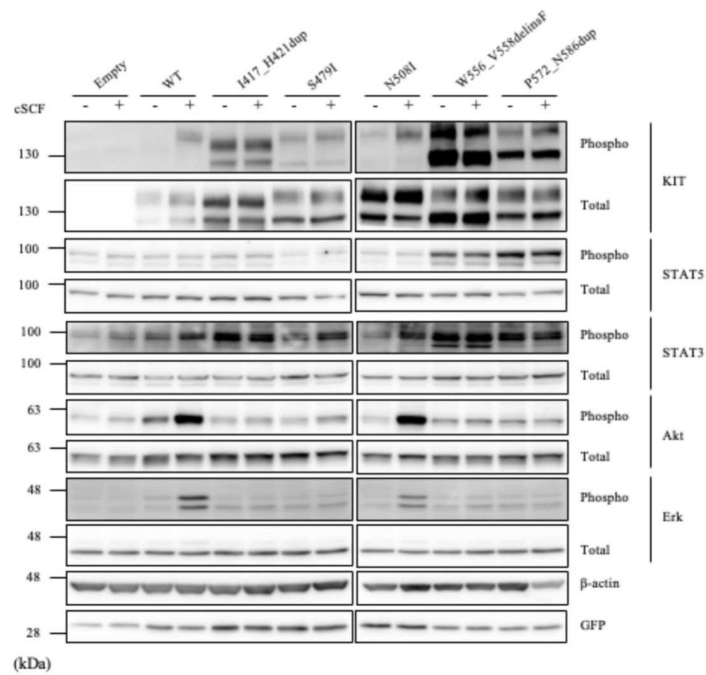


Figure S1. The phosphorylation of KIT and other signal proteins (STAT5, STAT3, Akt, and Erk) were analyzed by western blotting. β-actin was used as a loading control.

Table III-1. Primers used for construction of plasmid vectors.

Primer	Direction	Nucleotide sequence (5' to 3')
YTM178	F	CCACCGCCCTCAAAGTAGACG
YTM179	R	ACACCGGCCTTATTCCAAG
YTM2261	R	GATCTCCTCAACAACTGTACTTCATACAT
YTM2262	F	ATGTATGAAGTACAGTTTGTGAGGAGATC
YTM2263	R	ATGAGTCAGGATTTGAGTCAGGATTTCTGG
YTM2264	F	CAAATCCTGACTCATGAAAGTCTCACGAAT
YTM2794	F	GGTGGTACGGGAATTCGCCACCGCGATGAG
YTM2795	R	ATTTACGTAGCGGCCGCTCACACATCTTCG
YTM2812	F	ATAAGTGGGAATTCCTAGGAACAGGCTGAGCTTGGGAAAAC
YTM2814	R	GTTTCCTAGGGAATTCCTCACTTATGGTCATAGGGCAGTTGGGTCGG
YTM2815	F	CTAGTGGTTCAGATCTCCATCGATTAT
YTM2816	R	ATAATCGATGGAGATCTGAACCACTAG

F, forward; R, reverse

Table III-2. Primary antibodies used in this study.

Antibody	Clone	Type	Company	Dilution	Use for
pKIT	-	Rabbit polyclonal	Cell Signaling Technology	1:1000	Western blotting
KIT	-	Rabbit polyclonal	Dako	1:250	Immunohistochemistry
	2B8	Rat monoclonal	BioLegend	1:1000	Western blotting
			eBioscience	1:50	Immunocytochemistry
				1:160	Flow cytometry
pSTAT5	C71E5	Rabbit monoclonal	Cell Signaling Technology	1:1000	Western blotting
STAT5	A-9	Mouse monoclonal	Santa Cruz Biotechnology	1:1000	Western blotting
GM130	35	Mouse monoclonal	BD Bioscience	1:150	Immunocytochemistry
PDI	C81H6	Rabbit monoclonal	Cell Signaling Technology	1:100	Immunocytochemistry

Chapter 4

Establishment of a histiocytic sarcoma cell line and anti-tumor effect of proteasome inhibitors in the African pygmy hedgehog (*Atelerix albiventris*)

SUMMARY

The African pygmy hedgehog (*Atelerix albiventris*) is known to have a high incidence of tumor. However, investigating the tumors of this species has been constrained by the limited availability of research materials such as cell lines and genome information. In this study, I successfully established a novel cell line from a histiocytic sarcoma (HS) of an African pygmy hedgehog, allowing us to conduct a drug screening. I investigated using FDA-approved drug library screening to determine which anticancer drug this tumor cell line is sensitive to, and as a result of apoptosis experiments, bortezomib among the three proteasome inhibitors was found to induce cell death of cancer cells by significantly increasing caspase-3 cleavage ($p < 0.01$). Thus, I elucidated that the proteasome inhibitors, particularly bortezomib, exhibit anti-tumor effects on a cell line derived from an HS in an African pygmy hedgehog through a mechanism comparable to that described in human tumors. This study reports the first characterized cell line from the African pygmy hedgehog and also highlights the potential utility of bortezomib as an anti-tumor treatment for HS in this species.

INTRODUCTION

The African pygmy hedgehog (*Atelerix albiventris*), also known as the four-toed hedgehog, is a member of the order Eulipotyphla, family Erinaceidae, subfamily Erinaceinae (“Corrigendum: How many species of mammals are there?,” 2019), and is one of the most popular exotic pets in many countries, including Japan. This species is characterized by a high incidence of neoplastic disease. In several necropsy studies, 29%–60% of African pygmy hedgehogs were diagnosed with neoplastic disease (Raymond and White, 1999; Raymond and Garner, 2001; Heatley et al., 2005; Okada et al., 2018), and 74.6% were classified as malignant (Okada et al., 2018). In captivity, this species’ main cause of death is neoplastic disease, with a mortality rate of 35.9% (Pei-Chi et al., 2015). Therefore, basic and clinical research on neoplastic disease in African pygmy hedgehogs is necessary to elucidate the pathogenic molecular mechanisms and expand the development of treatment options.

Histiocytic sarcoma (HS) is a kind of malignant tumor common in dogs (Moore, 2014). In the African pygmy hedgehog, HS typically occurs in various organs, including the skin, abdominal organs, and nervous system, like the canine HS (Ogihara et al., 2016, 2017; Koizumi and Kondo, 2019; Koizumi et al., 2020). HS is classified as localized HS, disseminated HS (originates from interstitial dendritic cells, respectively), and

hemophagocytic HS (originates from bone marrow macrophages) (Moore, 2014). Each type of HS was reported in this species, and the clinical behavior and pathological features of HS in this species are similar to the canine HS (Makishima et al., 2021; Sirivisoot et al., 2021). A retrospective study on eight cases of African pygmy hedgehogs with HS showed that the median age at diagnosis was 2 years and 3 months, and the median survival time was 44 days (Son et al., 2020). However, there have been no reports of the clinical course of response to non-surgical treatment for HS in this species. To date, chemotherapy protocols have not been established for other tumors and HS in African pygmy hedgehogs, meaning that the cases with systemic metastases are not cured by surgical resection alone and have a poor prognosis. Thus, it is essential to search for effective anti-tumor drugs for HS and other types of tumors of this species. However, there has been no fundamental research on neoplastic disease in this species. This lack of studies is partly due to this species' lack of research materials, such as cell lines and genome information. Thus, establishing these basic materials and foundations is a top priority for researching African pygmy hedgehogs.

In this report, I established a new cell line derived from HS in an African pygmy hedgehog. Additionally, I put this cell line to use by searching for new anti-tumor drugs and analyzing their anti-tumor mechanism.

MATERIALS & METHODS

Ethics statement

The Ethics Review Board of the Joint Faculty of Veterinary Medicine of Yamaguchi University approved the study for a client's African pygmy hedgehog. The owner of the animal all agreed and provided written informed consent.

Cell line validation statement

The characteristics of the established cell line HHMYT in the present study were validated. The cell line was confirmed to be mycoplasma-free using a commercially available mycoplasma detection kit (iNtRON Bio, Burlington, MA, USA).

Cell line establishment

Primary HS tumor cells were obtained from the surgically removed mass of an African pygmy hedgehog for diagnostic and therapeutic purposes in Koizumi Nest Animal Hospital (Fukuoka, Japan) and were put in culture within 1 day post collection. Briefly, under sterile conditions and using a scalpel blade, the tumor tissue was mechanically dissociated into 1 mm small pieces. Minced tumor tissue was suspended in a warm DMEM-based primary culture medium (DMEM mixed with 20% fetal bovine

serum (FBS) and 100 units/ml penicillin-streptomycin) and transferred into a sterile 12-well tissue culture plate. Cultured primary cells were monitored daily using a microscope, and the medium was changed as required. After the 60th cell passaging, the medium was changed to RPMI1640-based complete medium (RPMI1640 mixed with 10% FBS, 100 units/mL penicillin-streptomycin and 55 μ M 2-mercaptoethanol) and passaging was continued at 37°C in a humidified CO₂ gas incubator.

I started the tumor cell culture, considered these cells to have stable traits and sustainable proliferation after over 100 passages, and eventually established a new HS cell line in African pygmy hedgehogs, which I named HHMYT. At the time of writing of this manuscript, single cell cloning had not been performed yet.

Reagents

An FDA-approved Drug Library, containing 1,134 compounds in fifteen 96-well microplates was kindly provided by Center for Supporting Drug Discovery and Life Science Research (Osaka University, Osaka, Japan). All compounds were at a concentration of 1 μ M in dimethyl sulfoxide (DMSO) solution.

Each proteasome inhibitor (namely bortezomib, carfilzomib, and disulfiram) was obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan),

Adipogen Life Sciences, Inc. (San Diego, CA), and Cayman Chemical Company (Ann Arbor, MI), respectively. Caspase inhibitor (Z-VAD-FMK) was obtained from the Peptide Institute (Osaka, Japan). Stock solutions of the above reagents were formulated in DMSO. All reagents were stored at -20°C .

Drug screening assay using an FDA-approved Drug Library

To screen the FDA-approved Drug library for cell growth inhibition, the HHMYT cell line was seeded at 2.0×10^4 cells in each single well of a 96-well plate containing 1 μM (final concentration) of each compound. DMSO was used in a control well.

In the experiments examining dose-dependency, the HHMYT cell line was seeded as above, containing compounds that showed growth inhibition to less than 50% by drug screening assay in the concentration range from 0 (control) to 10 μM (0, 0.1, 0.35, 1, 3.15, 10 μM).

After culture with the drug for 48 h, 10 μl of Cell Counting Kit-8 (DOJINDO Laboratories, Kumamoto, Japan) was added into each well. The plates were incubated for an additional 2 h at 37°C , and the absorbance at 450 nm was determined using a microplate reader (ARVO, PerkinElmer).

Cell proliferation and cytotoxicity assays

HHMYT cell line was seeded as in the above screening. Bortezomib and carfilzomib were added to each plate in the concentration range from 0 (control) to 200 nM (0, 3.125, 6.25, 12.5, 25, 50, 100, and 200 nM). Disulfiram was added to the plate in the concentration range from 0 (control) to 2400 nM (0, 37.5, 75, 150, 300, 600, 1200, 2400 nM). DMSO was added as a control. After 48 h of culture, I performed cell proliferation and cytotoxicity assays according to the previous report (Igase et al., 2020a). Additionally, in the caspase inhibition assay, HHMYT was incubated for 1 h in the presence of 50 μ M of Z-VAD-FMK before bortezomib was added to each well.

Annexin V apoptotic assay

To evaluate apoptosis and/or necrosis, HHMYT was seed at 5.0×10^5 cells/well in 6-well plates in the presence of bortezomib at 15.5 nM (the concentration corresponding to the IC_{50} value in cytotoxicity assay) or DMSO as a control. After 12 or 24 h of culture, cells were collected and washed in cold PBS and resuspended at 5.0×10^5 cells in 500 μ l of the $1 \times$ binding buffer from the PE Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA). Then, cells were incubated for 15 min at room

temperature in the dark after adding of 5 μ l each of PE Annexin V and 7-Amino-Actinomycin D (7-AAD), according to the manufacturer's instructions. The cells were detected using CytoFLEX (BECKMAN COULTER Life Science, Tokyo, Japan). Analyses were performed with FlowJo software.

Western blotting

To evaluate the expression of cleaved caspase-3, I performed the western blotting analysis according to the previous report (Igase et al., 2020a). HHMYT was seeded at 5.0×10^5 cells/well in 6-well plates in the presence of bortezomib, carfilzomib, or of disulfiram at concentrations corresponding to the IC₅₀ value in the cytotoxicity assay (15.5, 16.0, or 244.0 nM, respectively). DMSO was added as a control. Cells were collected and used for analysis after 12 or 24 h of culture. HHMYT was incubated for 1 h for caspase inhibition assay in the presence of 50 μ M of Z-VAD-FMK before starting each proteasome inhibitor treatment.

Immunocytochemistry

According to the previous report (Igase et al., 2020b), I conducted immunocytochemistry to identify the cell characteristics of the established cell line using

rabbit polyclonal anti-human Iba-1 antibody (Wako, diluted at 1:1,000) as first antibody and followed by incubation with DyLight 488-conjugated goat anti-rabbit IgG (Abcam). Rabbit IgG isotype antibody (clone DA1E, Cell Signaling Technology, Inc., Massachusetts, U.S.) was used as a negative immunostaining control. Samples were visualized at BZ-X800 (KEYENCE, Osaka, Japan).

Statistical analysis

The Tukey–Kramer test and Student’s t-test determined the significance of differences between samples. Multiple comparison test was performed using JMP Pro 15 software (SAS Institute Japan, Tokyo, Japan). Statistical significance was set at $p < 0.05$.

RESULTS

Identification of an African pygmy hedgehog with HS

A 3-year-old male African pygmy hedgehog was diagnosed with HS in the right forelimb. This case underwent surgery at Koizumi Nest Animal Hospital (Fukuoka, Japan), and the surgically resected tumor tissue was examined pathologically in Nihon university (Kanagawa, Japan). A piece of tumor tissue was obtained to establish a cell line before formalin fixation. Histopathologically, an infiltrative mass with indistinct borders is located from the dermis to the subcutaneous tissue. The mass is composed of interlocking or sheet-like proliferations of spindle-shaped to rounded cells. The tumor cells have indistinct cell borders and a small amount of acidophilic cytoplasm. The nuclei are of unequal size and round, with coarse chromatin and well-defined nucleoli. The atypia is marked, and the mean number of mitotic figures was 2.7 per HPF. Immunohistochemistry showed that most of the tumor cells expressed Iba-1 and some cells expressed HLA-DR as well as Iba-1. Based on these histopathological and immunohistochemical findings, this case was diagnosed as localized HS. In this case, a recurrence was found at the same site 5 months after the surgery, and the animal underwent a second excision. The outcome of the case was unknown at the time point of the writing of the manuscript.

Immunocytochemical validation of the HHMYT cell line

To test if the HHMYT cell line is derived from HS, its morphological characteristics and expression of Iba-1 were examined using Diff-Quik staining and immunocytochemistry (Figure IV-1). The morphology of HHMYT is spindle-shaped to rounded cells, similar to that of primary tumor tissue, which consists of spindle-to-rounded tumor cells. Iba-1 was found to be expressed in the HHMYT cell line as well as in primary tumor tissue.

Proteasome inhibitors inhibit cell proliferation and induce cell death of HHMYT

To identify a novel anti-tumor drug suppressing the cell proliferation of the HS cell line from an African pygmy hedgehog, a drug screening assay using the FDA-approved Drug Library (1 μ M of each compound) was performed using the HHMYT cell line. Several compounds suppressed the cell proliferation of HHMYT (Figure IV-2A and Table IV-1). Next, to evaluate anti-tumor effects in a dose-dependent manner, I further performed a second screening assay using the top 18 compounds that showed less than 50% growth inhibition in the first drug screening assay. Based on the data from these compounds (Figure IV-2B), I focused on proteasome inhibitors (bortezomib and

carfilzomib) and disulfiram, an anti-alcoholism drug, which is also known to have anti-tumor effect through inhibition of the proteasome (Lu et al., 2021).

First, to confirm the suppressive effects of proteasome inhibitors on cell proliferation under a broader range of compound concentrations, a cell proliferation assay was performed using the HHMYT cell line. Cell proliferation of HHMYT was inhibited by three proteasome inhibitors in a dose-dependent manner (Figure IV-3A–C). The IC_{50} values of each inhibitor (bortezomib, carfilzomib, and disulfiram) for HHMYT were 10.2, 13.3, and 241.0 nM, respectively.

Next, I examined the cytotoxic effect of proteasome inhibitors on HHMYT. The cytotoxicity of the three inhibitors was observed in a dose-dependent manner (Figure IV-3D–F). The IC_{50} values of each inhibitor (bortezomib, carfilzomib, and disulfiram) for HHMYT were 15.5, 16.0, and 244.0 nM, respectively. These results indicated that proteasome inhibitors induced the cell death in the HHMYT cell line.

Proteasome inhibitors induce apoptosis by activating caspase-3 in HHMYT

To assess the mechanism of cell death induced by proteasome inhibitors in HHMYT, caspase-3 cleavage was examined using western blotting analysis. Cleaved caspase-3 protein was detected in HHMYT 12 h after treatment with IC_{50} values of all

proteasome inhibitors (Figure IV-4A). Among them, caspase-3 cleavage increased significantly in the cells treated with bortezomib ($p < 0.01$). Based on this result, I focused on bortezomib in the subsequent experiments.

Cells were stained with Annexin V and 7-ADD, and analyzed by flow cytometry. Early-apoptosis and late-apoptosis cells were increased in the HHMYT cell line 12 to 24 h after bortezomib treatment (Figure IV-4B).

Next, to confirm whether proteasome inhibitors induced cell death via caspase activation, HHMYT cells were pre-treated with the pan-caspase inhibitor Z-VAD-FMK before bortezomib treatment. Cytotoxicity assay using trypan blue dye showed that bortezomib-induced cell death was significantly inhibited by adding Z-VAD-FMK in the HHMYT cell line (Figure IV-5A). Moreover, as expected, adding Z-VAD-FMK suppressed bortezomib-induced caspase-3 cleavage in the HHMYT cell line (Figure IV-5B). However, bortezomib-induced cell death was not completely inhibited by treatment with Z-VAD-FMK. These results suggested that bortezomib-induced cell death involves another mechanism in addition to caspase activation in the HHMYT cell line.

DISCUSSION

This is the first report about establishing a tumor cell line from an African pygmy hedgehog HS. In addition to establishing the cell line, I showed that proteasome inhibitors have anti-tumor effects on the HHMYT cell line by inducing apoptosis. The proteasome is a large multi-subunit protease complex that degrades ubiquitinated proteins in an ATP-dependent manner (Finley, 2009). It regulates various cellular events, such as cell cycling, cell death, signal transduction, and differentiation, by degrading proteins involved in these functions and misfolded and mutated proteins (Ciechanover, 1994; Goldberg, 2003). In humans, proteasome inhibitors induce the accumulation of misfolded proteins and endoplasmic reticulum stress by inhibiting the chymotrypsin-like activity in the proteasome (Nawrocki et al., 2005), leading to apoptosis of tumor cells (Hideshima et al., 2001). The activation of the caspase pathway induces the apoptosis associated with endoplasmic reticulum stress via several mechanisms, such as C/EBP homologous protein (Ma et al., 2002), death receptor 5 protein (Yamaguchi and Wang, 2004), and JNK pathway (Yoneda et al., 2001). In addition, proteasome inhibitors suppress the activation of nuclear factor-kappa B (NF- κ B). Suppression of NF- κ B leads to inhibition of cell proliferation via suppression of the expression of cytokines such as IL-6 and TNF- α (Adams, 2004). Based on these mechanisms for anti-tumor effect, bortezomib has been

approved for multiple myeloma (Mateos et al., 2010), mantle-cell lymphoma (Robak et al., 2015), and, Waldenström macroglobulinemia (Chen et al., 2007), and carfilzomib for multiple myeloma (Hájek et al., 2012; Dimopoulos et al., 2020). In the present study, I found that bortezomib-induced apoptosis is dependent on the caspase pathway and that carfilzomib and disulfiram induced the cleavage of caspase-3. These results suggest that proteasome inhibitors, particularly bortezomib, have anti-tumor effects on African pygmy hedgehogs HS by a mechanism similar to that found in humans.

Interestingly, bortezomib is also approved for subcutaneous injection in human medicine and has achieved anti-tumor effects comparable to its intravenous injection (Moreau et al., 2011, 2015). In veterinary medicine, African pygmy hedgehogs are known to be cautious animals, making placing an intravenous line in a non-sedated condition difficult. Thus, in such animals, bortezomib could be a highly beneficial agent because it can be injected subcutaneously. Future studies are needed to confirm the clinical usefulness of bortezomib, including safety and pharmacokinetic tests in African pygmy hedgehogs.

This study has several limitations. First, only one cell line was established and used in the assays. Therefore, it is unclear whether these data represent HS in African pygmy hedgehogs. Further establishment of HS cell lines and evaluation of drug

sensitivity using those cell lines are needed. Second, I did not analyze the detailed apoptosis pathway except for the cleavage of caspase-3. This is because very few antibodies are available for this species. Thus, the genome sequence of this species must be elucidated to select appropriate antibodies and analyze in more detail the apoptosis pathways.

In conclusion, proteasome inhibitors induce apoptosis via the caspase pathway in the HS cell line HHMYT derived from an African pygmy hedgehog. Future studies are needed to investigate the clinical usefulness of proteasome inhibitors, particularly bortezomib. In addition, the development of basic research on tumors in African pygmy hedgehogs is expected.

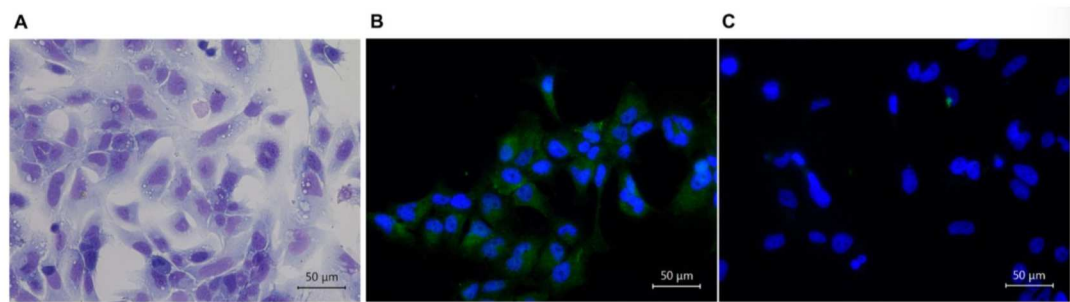


Figure IV-1. Results of Diff-Quik staining (A) and immunocytochemistry for Iba-1 (B) and isotype control (C) in the HHMYT cell line. Scale bar, 50 μm.

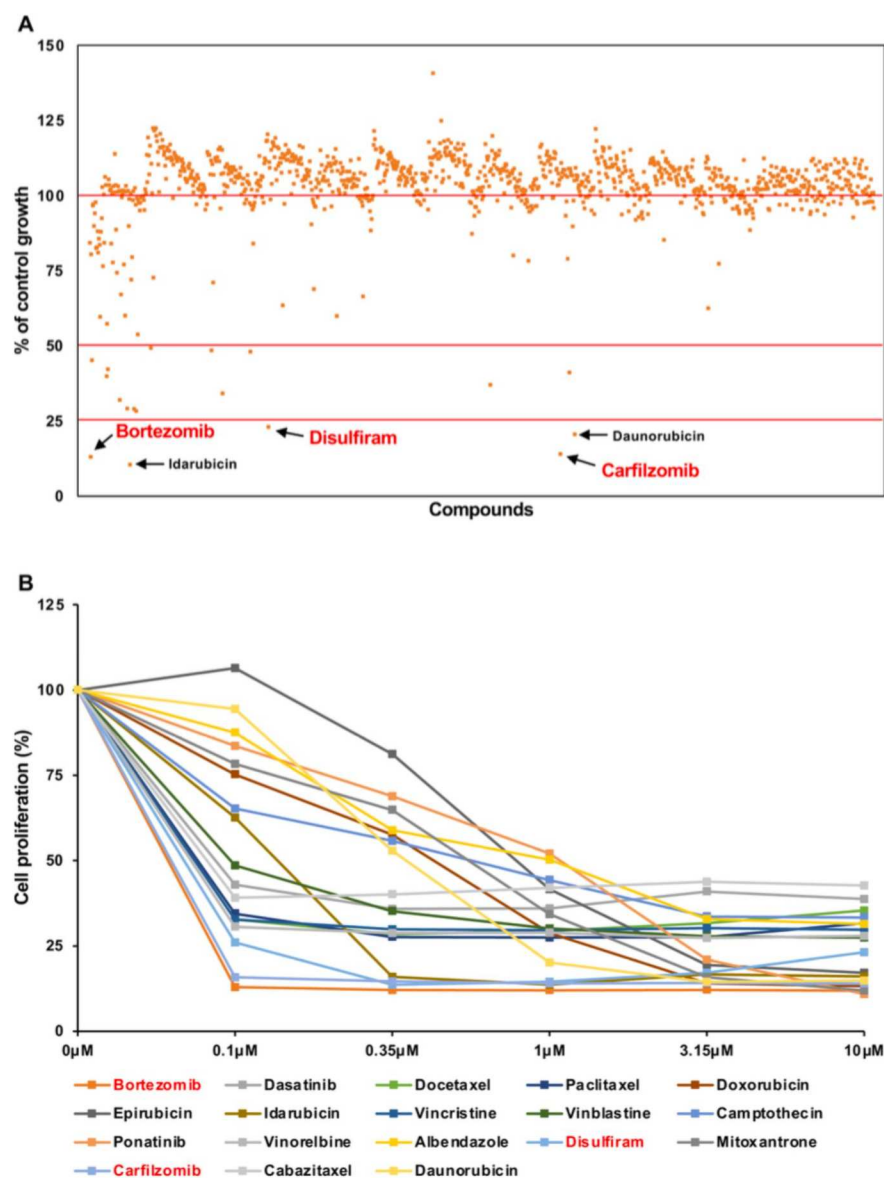


Figure IV-2. (A) Results of drug screening assay using the FDA-approved Drug Library (including 1,134 drugs) in HHMYT cell line. The y-axis represents cell survival, and each dot represents the result for each compound. The results of proteasome inhibitors are labeled with the names of the compounds. (B) The results of the second screening focused on the top 19 compounds that showed growth inhibition to less than 50% in the first screening. The y-axis represents cell survival.

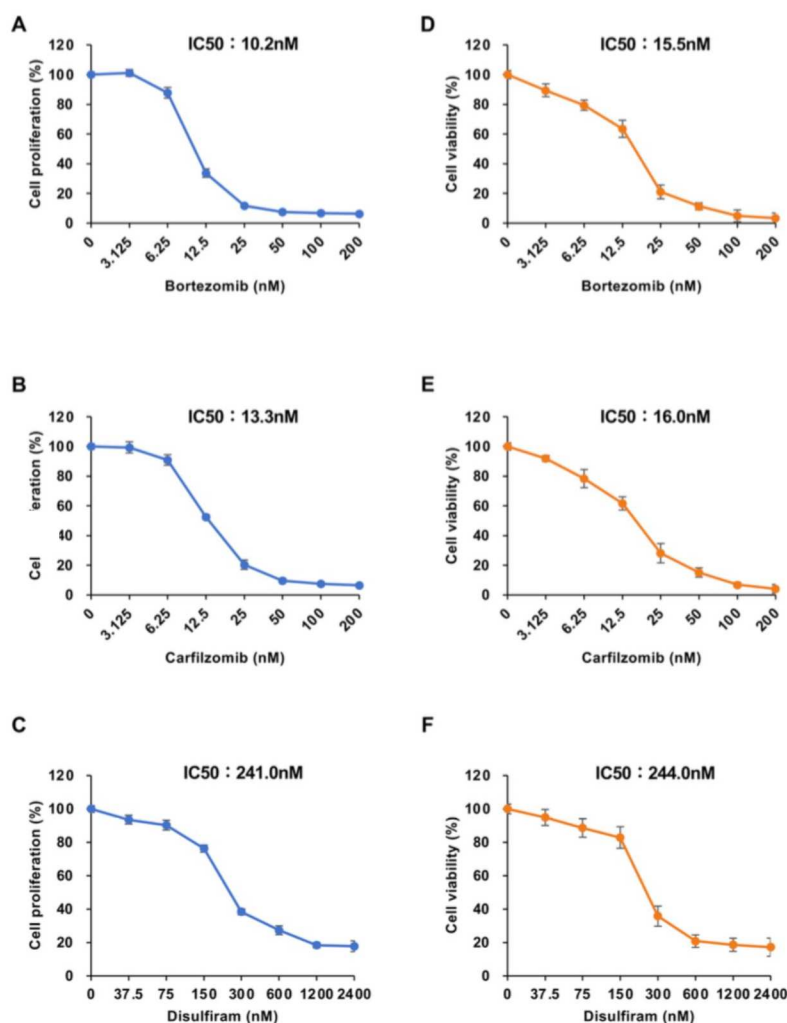


Figure IV-3. Results of cell proliferation (A-C) and cytotoxicity (D-F) assays using proteasome inhibitors. The HHMYT cell line was seeded in the presence of bortezomib (A, D), carfilzomib (B, E), or disulfiram (C, F) at the indicated concentration in triplicates for 48h, and the cell proliferation rate and cell viability were counted by Cell Counting Kit-8 reagent and trypan blue exclusion test, respectively. The y-axis indicates the proliferation ratio or the viability of drug-treated cells at each concentration when DMSO-treated cells were set as 100%. The results were expressed as mean $\pm$ SD of three independent experiments.

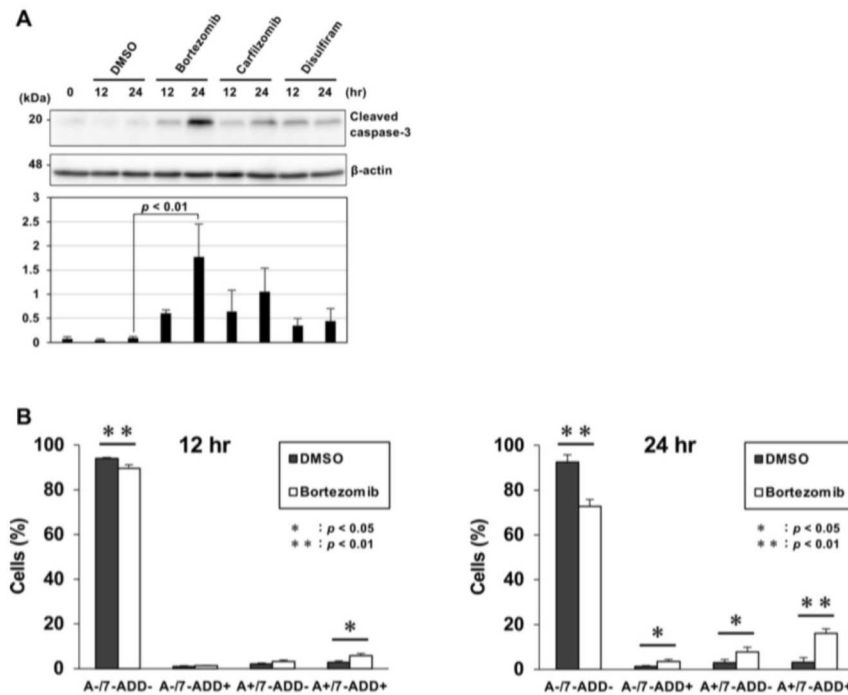


Figure IV-4. (A) Analysis of caspase-3 cleavage by western blotting. The HHMYT cell line was cultured for 12 and 24h in the presence of each proteasome inhibitor (bortezomib, carfilzomib, or disulfiram), and the proteins were extracted for western blotting using anti-cleaved caspase-3 antibodies. Beta-actin was used as a protein loading control. Each blot represents one of three experiments showing a similar trend. The graphs below each image quantify the expression level of the densitometry analysis of the target protein band relative to the beta-actin band using ImageJ (National Institute of Health, Bethesda, MD). The results were expressed as mean $\pm$ SD of three independent experiments. Tukey-Kramer test. (B) Results of Annexin V/7-ADD assay. The HHMYT cell line was cultured for 12 and 24h in the presence of bortezomib and stained with Annexin V/7-ADD before flow cytometry analysis. The cell population of apoptotic and necrotic cells was analyzed by Annexin V/7-ADD assay. The results were expressed as mean $\pm$ SD of three independent experiments. Student's *t*-test. *, $P < 0.05$; **, $P < 0.01$

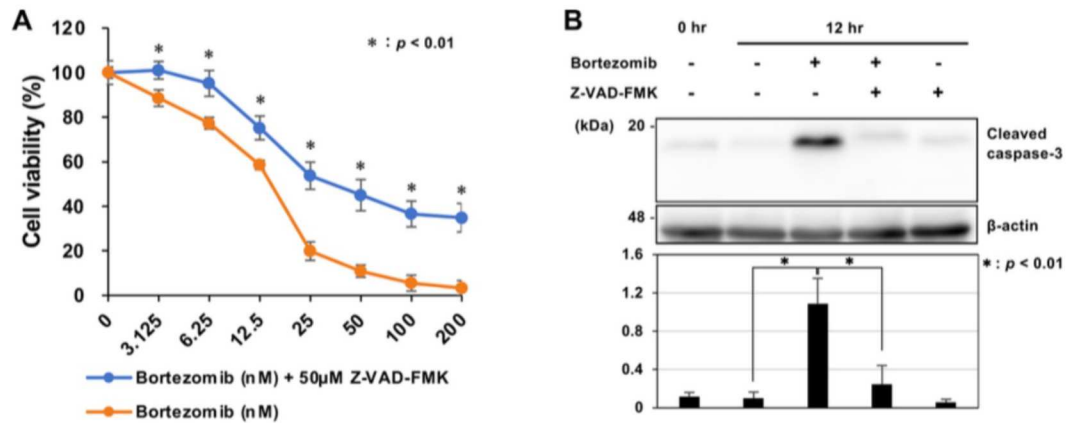


Figure IV-5. Results of caspase inhibition assay. The HHMYT cell line was cultured in the presence of bortezomib with or without caspase inhibitor Z-VAD-FMK (50 μ M), followed by the analysis of cell viability using trypan blue exclusion test (A) and the analysis of caspase-3 cleavage by western blotting (B). (A) After 48h of culture, the live or dead cells were counted with 0.25% trypan blue, y-axis indicates the viability of drug-treated cells at each concentration when DMSO-treated cells were set as 100%. (B) After 12h of culture, the proteins were extracted for western blotting using anti-cleaved caspase-3 antibodies. Beta-actin was used as a protein loading control. Each blot represents one of three experiments showing a similar trend. The graphs below each image quantify the expression level of the densitometry analysis of the target protein band relative to the beta-actin band using ImageJ. The results were expressed as mean $\pm$ SD of three independent experiments. Tukey-Kramer test. *, $P < 0.05$

Table I-1. Drug screening by FDA-approved Drug Library in histiocytic sarcoma cell line in African pygmy hedgehog. Because the library comprises 1,134 drugs, the full table are presented at the end of this thesis for clarity and readability. (shown in the final section of this dissertation.)

Chapter 5

Functional analysis of tumor suppressor protein p53 in the African pygmy

hedgehog (*Atelerix albiventris*)

SUMMARY

Peto's paradox describes the observation that tumor incidence does not scale with body sizes or lifespan across species, implying the existence of species-specific tumor-suppressive mechanisms. Whereas previous work has focused mainly on large and/or long-lived mammals, the mechanisms underlying high tumor incidence in small-bodied species remains poorly understood. The African pygmy hedgehog (*Atelerix albiventris*) is characterized by a strikingly high prevalence of neoplastic disease, yet the endogenous factors contributing to this susceptibility have not been clarified. In this study, I analyzed a draft genome assembly of the African pygmy hedgehog and identified marked structural divergence in its *TP53* gene, particularly within the transactivation domain (TAD1, TAD2) and proline-rich domain (PRD). Functional assays using recombinant p53 constructs demonstrated that *A. albiventris* p53 (Afh-p53) induced significantly weaker apoptosis than human-p53 in both human and African pygmy hedgehog-derived cell lines, while retaining a comparable capacity to induce cell cycle arrest. Afh-p53 exhibited reduced transactivation of pro-apoptotic targets, and this defect was rescued by chimeric constructs in which the human TAD/PRD regions were introduced into Afh-p53. Structural modeling predicted similar DNA-binding properties across species, whereas molecular docking suggested diminished interactions of Afh-p53

with SH3-domain proteins such as ASPP1, in consistent with a reduced number of PXXP motifs in its PRD. These findings support the notion that African pygmy hedgehogs retain a more ancestral-like *TP53* with attenuated apoptotic function, which may represent one molecular contributor to their high tumor incidence. This study highlights value of studying small bodied species and provides new insight into the evolutionary diversity of mammalian tumor-suppressive strategies.

INTRODUCTION

Peto's paradox refers to the observation that tumor incidence does not show the proportions with body sizes or lifespans across species (Peto et al., 1975; Peto, 2016). Although large, long-lived animals such as whales and elephants possess far more cells and undergo many more lifetime cell divisions than small and/or short-lived animals, they do not exhibit proportionally higher rates of tumors (Abegglen et al., 2015; Nunney et al., 2015; Boddy et al., 2020). These counterintuitive findings may be explained by enhanced tumor-suppression mechanisms, such as increased tumor-suppressor gene copies, reduced oncogene activities, and species-specific alterations in metabolic rates viral susceptibilities, and immune surveillance (Caulin and Maley, 2011). For instance, elephants (*Loxodonta africana*)—with large body sizes and lifespans of up to 70 years (de Magalhães and Costa, 2009)—have expanded the copy number and function of *TP53* gene in proportion to body size (Abegglen et al., 2015; Sulak et al., 2016; Padariya et al., 2022). Naked mole-rats (*Heterocephalus glaber*)—which live for 28 years in wild and rarely develop tumors (Buffenstein and Jarvis, 2002; Buffenstein, 2005)—exhibit an increased sensitivity to contact inhibition in their cells (Seluanov et al., 2009). Bowhead whales (*Balaena mysticetus*), which can live for more than 200 years (George et al., 1999), show positive selections in several tumor-associated genes (Keane et al., 2015).

These reports on Peto's paradox have focused primarily on large and/or long-lived animals, advancing our understanding of tumor-suppressive mechanisms that have evolved in these species. In contrast, studies that address the tumorigenesis in small and/or short-lived animals remain extremely limited.

The African pygmy hedgehog (*Atelerix albiventris*), also known as the four-toed hedgehog, belongs to the order Eulipotyphla, family Erinaceidae, subfamily Erinaceinae (Burgin et al., 2025). It is one of the most popular exotic pets in many countries, including Japan. This species is characterized by a high incidence of neoplastic disease. In several necropsy studies, 29%–60% of African pygmy hedgehogs were diagnosed with neoplastic disease (Raymond and White, 1999; Raymond and Garner, 2001; Heatley et al., 2005; Okada et al., 2018), of which 74.6% were classified as malignant (Okada et al., 2018). In captivity, neoplastic disease is the leading cause of death, accounting for 35.9% of mortality cases (Pei-Chi et al., 2015).

In reports on tumor prevalence across vertebrates, limited species exhibited equivalent rates to African pygmy hedgehogs, such as ferrets (*Mustela putorius*; 63%), opossums (*Didelphis marsupialis*; 56%), Baltic gray seals (*Halichoerus grypus*; 64%), South African Cape Mountain zebras (*Equus zebra*; 53%), and white suckers (*Catostomus commersonii*; 59%) (Bäcklin et al., 2003; Marais et al., 2007; Coffee et al., 2013;

Madsen et al., 2017; Compton et al., 2025). In these species, factors such as environmental pollutions, genetic vulnerabilities, and hormonal abnormalities have been suggested as potential contributors to tumorigenesis (Smith et al., 1989; Bergman, 1999; Hoppes, 2010). These observations suggest that the underlying factors contributing to tumorigenesis may differ substantially among species even when tumor incidence is similarly high. In addition, studies of captive mammals and sauropsids reported that the mean age at death for tumor-bearing cases is lower than the average lifespan of those without tumors, indicating that tumor is not merely a due to extended lifespan (Compton et al., 2025). Taken together, there may be endogenous factors deciding susceptibility to tumors and exogenous environmental factors (Ames, 1989; Hussain and Harris, 2000); however, it has been unclear that why tumors develop frequently in this small and medium lifespan species equivalent with human and dogs.

In this study, I sought to identify endogenous factors that may underlie the unusually high tumor incidence in African pygmy hedgehogs. Using a draft genome assembly, I first annotated tumor-related genes and focused on the unique structural features of the hedgehog *TP53* gene. I then examined how these genomic characteristics influence p53-mediated tumor-suppressive functions by combining *in vitro* functional assays in human and hedgehog-derived cell lines with *in silico* structural and docking

analyses. Through this integrative approach, I aimed to clarify how species-specific properties of African pygmy hedgehog *TP53* may contribute to tumor susceptibility and to provide new insight into the evolutionary diversity of mammalian tumor-suppressive strategies.

MATERIALS & METHODS

Ethics statement

The Ethics Review Board of the Joint Faculty of Veterinary Medicine of Yamaguchi University approved the study for a client's African pygmy hedgehog. The owner of the animal all agreed and provided written informed consent.

Cell culture

The retroviral packaging cell line Platinum-GP (Plat-GP) was kindly provided by Dr. Toshio Kitamura (The University of Tokyo, Tokyo, Japan). The human embryonic kidney cell line HEK293T (RCB2202) was obtained from the RIKEN BioResource Research Center (RIKEN BRC, Ibaraki, Japan). The human *TP53*-null osteosarcoma cell line Saos-2 (TKG-0469) was obtained from the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan), and the human *TP53*-null non-small cell lung cancer cell line NCI-H1299 (CRL-5803) was obtained from the American Type Culture Collection (ATCC). Plat-GP and HEK293T cell lines were cultured in n DMEM supplemented with 10% fetal bovine serum (D10 complete medium). Saos-2, H1299 and the African pygmy hedgehog-histiocytic sarcoma cell line HHMYT (Inanaga et al., 2024) were cultured in RPMI1640 supplemented with 10% fetal bovine serum (R10 complete

medium). All cell lines were maintained at 37 °C in a humidified CO₂ gas incubator.

Establishment of a tumor cell line in African pygmy hedgehogs

Primary granulosa cell tumor (GCT) cells were obtained from a surgically excised mass of an African pygmy hedgehog that underwent surgery for diagnostic and therapeutic purposes in Koizumi Nest Animal Clinic (Fukuoka, Japan), and were placed in culture within 1 day of collection. Under sterile conditions, the tumor tissue was mechanically dissociated with a scalpel into ~1 mm small pieces. The minced tumor tissue was suspended in pre-warmed D20 complete medium and transferred to sterile 12-well tissue culture plates. primary cultures were monitored daily by microscopy, and the medium was changed as required. After 60 passages, the culture medium was switched to R10 complete medium, and passaging was continued at 37°C in a humidified CO₂ incubator. When the cells had undergone more than 100 passages and exhibited stable characteristics with sustained proliferation, the culture was considered established as a new GCT cell line from an African pygmy hedgehog, which we named HHOVT.

Annotation of tumor-associated genes in African pygmy hedgehogs

A draft genome assembly of the African pygmy hedgehog (*Atelerix albiventris*)

was obtained from Project Kalunguyeye. Tumor-associated genes were annotated by lifting over gene models from the Western European Hedgehogs (*Erinaceus europaeus*; assembly eriEur1) using Liftoff v1.6.3. (Shumate and Salzberg, 2021).

Alignment of amino acid sequences

TP53 orthologs and the corresponding p53 amino acid sequences from vertebrate species listed in Table V-1 were retrieved from Ensemble and Uniprot. The amino acid sequences were aligned using Clustal Omega (EMBL-EBI online version). The resulting multiple sequence alignment was then used to construct a phylogenetic tree representing the evolutionary relationships among p53 orthologs. Default parameters were used unless otherwise specified.

Reagents

The doxycycline and doxorubicin were obtained from Takara Bio Inc. (Shiga, Japan) and Thermo Fisher Scientific K.K. (Tokyo, Japan), respectively. Stock solutions of these reagents were formulated in milliQ and stored at –20 °C.

Molecular cloning of TP53 cDNAs derived from African pygmy hedgehog TP5

Total RNAs were isolated from the HHMYT and HHOVT cell lines using the ISOGEN II kit (NIPPON GENE, Co, Ltd., Tokyo, Japan), and cDNAs were synthesized using Superscript III first-strand synthesis system for RT-PCR (Thermo Fisher Scientific K.K.), according to the manufacturer's instructions. *TP53* cDNAs from African pygmy hedgehog were amplified by PCR. The resultant PCR products were electrophoresed in agarose gel, and the expected size of band was extracted, followed by second PCR. The nucleotide sequence analysis was performed by the DNA Core Facility of the Center for Gene Research, Yamaguchi University.

CRISPR-Cas-mediated knockout of *AfhTP53*

According to the previous report (Igase et al., 2019), CRISPR-Cas9 small guide RNAs (sgRNA) targeting the *AfhTP53* gene, were designed using an online CRISPR design tool (<http://zlab.bio/guide-design-resources>) Two complementary oligonucleotides sequences of sgRNA of *AfhTP53* were synthesized: YTM2495, 5'-CACCGAGAAACCTTTACCGACTTCC-3'; and YTM2496, 5'-AAACGGAAGTCGGTAAAGGTTTCTC-3'. The oligonucleotides were annealed and ligated into the BsmBI-digested lentiCRISPR v2 plasmid (a gift from Dr. Feng Zhang; Addgene plasmid #52961; <http://n2t.net/addgene:52961>; RRID: Addgene 52961) to

generate lentiCRISPR-ko-Afhp53#2. lentiCRISPR-ko-Afhp53#2 was then subcloned into a hygromycin-resistant selection vector by restriction enzyme digestion and ligation, yielding lentiCRISPR-H-ko-Afhp53#4. To produce lentivirus-expressing the *TP53*-targeting sgRNA, plasmids were transfected into the HEK293T cell line using Lipofectamin®3000 (Thermo Fisher Scientific K.K.). After 24h, the supernatant of HEK293T cells was replaced with a D10 complete medium without antibiotics. After another 24h, the supernatant containing lentiviruses was collected and transferred onto HHOVT cells. After 12h of infection, the supernatant was replaced with a D10 complete medium, followed by the addition of 1.0 mg/ml hygromycin (InvivoGen, San Diego, CA) onto the HHOVT cells to select stably transduced cells. Individual single clones were isolated by limited dilution method and further analyzed by western blotting to confirm loss of p53 protein expression.

Tet-on-inducible TP53 expression

To generate Tet-on-inducible *TP53*-expressing cells, a series of retroviral plasmids were constructed as follows. The Zeocin-resistant Tet-On retroviral plasmid pRetroX-Tet-On-Advanced-IZ #7 was generated by excising the Zeocin resistance cassette from the retroviral vector pMx-IZ #7 (laboratory stock) by EcoRI and SalI

digestion, followed by ligation of the EcoRI–SalI fragment into pRetroX-Tet-On-Advanced (Clontech) digested with XhoI and EcoRI.

The human *TP53* expression plasmid pRetroX-TP-hp53#1 was constructed by PCR amplification of human *TP53* cDNA from pCAGhp53WT (RDB02429, RIKEN BRC) using primers YTM2499 and YTM2450. The PCR product and pMXs-IP were digested with EcoRI and XhoI and ligated to generate pMx-IP-hp53#7. The BamHI–NotI fragment containing the *hTP53* insert from pMx-IP-hp53 #7 was then subcloned into pRetroX-Tight-Pur (Clontech) digested with BamHI and NotI, yielding pRetroX-TP-hp53 #1.

The African pygmy hedgehog *TP53* expression plasmid pRetroX-TP-Afhp53 #7 was constructed as follows. *AfhTP53* cDNA was amplified from HHMYT cell line–derived cDNA using primers YTM2477 and YTM2478 and ligated into the SmaI site of pBluescript SK (–) to obtain pBS-AfhTP53-HHMYT#7. The *AfhTP53* insert was excised from pBS-AfhTP53-HHMYT#7 by EcoRI and NotI digestion and ligated into the EcoRI–NotI sites of pMXs-IP to generate pMx-IP-Afhp53#1. The *AfhTP53* fragment was then excised from pMx-IP-Afhp53#1 with EcoRI and NotI and subcloned into CSII-EF-cPDL1-IP#4 digested with EcoRI and NotI, yielding CSII-EF-IP-Afhp53#1. An *AfhTP53* fragment was subsequently amplified from CSII-EF-IP-Afhp53 #1 with primers

YTM2550 and YTM2551 and inserted into pRetroX-Tight-Pur linearized by BamHI–EcoRI digestion using an In-Fusion cloning kit (Takara), resulting in pRetroX-TP-Afhp53 #7.

The chimeric *hhh-AfhTP53* plasmid pRetroX-TP-hhh-Afhp53 #13 was generated as follows. A human *TP53* fragment was amplified from pMx-IP-hp53#7 using primers YTM178 and YTM2505, and an *AfhTP53* fragment was amplified from pMx-IP-Afhp53#1 using primers YTK206 and YTM2506. These two PCR products were fused by overlap-extension PCR with primers YTM178 and YTK206. The resulting EcoRI–NotI-flanked fragment was digested with EcoRI and NotI and ligated into CSII-EF-fPDL1-IP#4 digested with EcoRI and NotI to obtain CSII-EF-hhh-Afhp53#1. The *hhh-Afhp53* insert was then amplified from CSII-EF-hhh-Afhp53#1 using primers YTM2552 and YTM2551 and cloned into pRetroX-Tight-Pur linearized with BamHI and EcoRI by In-Fusion cloning to generate pRetroX-TP-hhh-Afhp53#13.

For the construction of pRetroX-TP-hha-Afhp53 #1, intermediate Tet-One plasmids were first generated. A *hTP53* fragment was amplified from pMx-IP-hp53#7 using primers YTM2619 and YTM2620, and the fragment was amplified from pMx-IP-PDL1-FL#9 using primers YTM2621 and YTM2622. These two fragments were fused by overlap-extension PCR with primers YTM2619 and YTM2622, and the resulting

product was inserted by In-Fusion cloning into pRetroX-TetOne-Puro (Takara/Clontech) linearized with BamHI and EcoRI, yielding pRetroX-TetOneP-hp53#7. For the *AfhTP53* Tet-One plasmid, two overlapping *AfhTP53* fragments were amplified from pMx-IP-Afhp53 #1 using primer pairs YTM2624/YTM2627 and YTM2628/YTM2625, fused by overlap-extension PCR with primers YTM2624 and YTM2625, and combined with a fragment amplified from pMx-IP-fPDL1-FL#9 using primers YTM2626 and YTM2622 by overlap-extension PCR with primers YTM2624 and YTM2622. The final product was cloned by In-Fusion into BamHI–EcoRI-digested pRetroX-TetOne-Puro to obtain pRetroX-TetOneP-Afhp53#7.

To generate pRetroX-TP-hha-Afhp53#1, a fragment was amplified from pRetroX-TetOneP-Afhp53#7 using primers YTM2622 and YTM2634, and another fragment was amplified from pRetroX-TetOneP-hp53#7 using primers YTK2619 and YTM2635. These fragments were fused by overlap-extension PCR with primers YTK2619 and YTM2622 and further amplified with primers YTM2695 and YTM2697. In parallel, a backbone fragment was amplified from pRetroX-TP-Afhp53 #7 using primers YTM2710 and YTM2711. The *hha-Afhp53* insert and the pRetroX-TP-Afhp53 #7–derived backbone were recombined by In-Fusion cloning to yield pRetroX-TP-hha-Afhp53 #1.

The *hah-Afp53* plasmid pRetroX-TP-hah-Afp53#1 was generated by assembling four overlapping fragments. A fragment was amplified from pRetroX-TetOneP-hp53#7 using primers YTM2619 and YTM2638, a second fragment from pRetroX-TetOneP-Afp53#7 with primers YTM2639 and YTM2640, a third fragment from pRetroX-TetOneP-hhh-Afp53#13 with primers YTM2605 and YTM2641, and a fourth fragment from pRetroX-TetOneP-Afp53#7 with primers YTM2506 and YTM2622. These four fragments were fused by overlap-extension PCR using primers YTM2619 and YTM2622. The resulting chimeric PCR product was further amplified with primers YTM2695 and YTM2697. In parallel, a backbone fragment was amplified from pRetroX-TP-Afp53#7 using primers YTM2710 and YTM2711. The final *hah-Afp53* insert was recombined with the pRetroX-TP-Afp53 #7 backbone by In-Fusion cloning to obtain pRetroX-TP-hah-Afp53 #1.

To establish Tet-on inducible *TP53*-expressing cell lines, a pRetroX-Tet-On-Advanced-IZ #7 plasmid were first transfected into the Plat-GP cell line using Lipofectamin®3000. Using the same procedure performed for generating the knockout cells, pRetroX-Tet-On-Advanced-IZ #7 was transduced into Saos-2, H1299, and HHOVT cells. After 12h of infection, the supernatant was replaced with a D10 complete medium, followed by the addition of 100 µg/ml Zeocin (InvivoGen) onto cells to select stably

transduced cells. Next, each TP53 plasmid was transfected into the Plat-GP cell line using Lipofectamin®3000, followed by the same procedure as above to transduce each *TP53* construct into cells. After that, 1.25 µg/ml of puromycin (InvivoGen) was added onto cells.

Cell proliferation assay

Each cell line was seeded into 96-well plate at 5000 cells/well. Doxycycline was added to each well at a concentration of 1 µM. After incubation for indicated time points (0, 24, 48, and 72h), the CCK8 was added to each well, and the cells were incubated for 3h at 37°C. The HHOVT cell line was seed into 96-well plate at 5000 cells/well. Doxycycline was added to each well in 1 µM and doxorubicin was added in the concentration range from 0 (control) to 1.0 µM. After 48h of culture, the CCK8 was added to each well, and the cells were incubated for 3h at 37°C. The absorbance was measured at 450 nm by VarioSkan LUX (Thermo Fisher Scientific K.K.). All experience were performed three times in each cell line.

Cell cytotoxicity assays

Each cell line was seeded into 96-well plate at 5,000 cells/well. Doxycycline

was added to each well at a concentration of 1 μ M. After incubation for indicated time points (24, 48, and 72h), the trypan blue exclusion test was performed in each well according the previous report (Inanaga et al., 2024).

Cell cycle analysis

Each cell line was seeded into 12-well plate at 2.0×10^5 cells/well in the presence of 1 μ M doxycycline. After 24h, the cells were collected and washed with phosphate-buffered saline (PBS), resuspended in 70% ice-cold ethanol, and incubated at 4 °C for >2h. The cells were treated with 100 μ g/ml of RNase (Nacalai Tesque, Kyoto, Japan) at room temperature for 30 min, and followed by addition of 40 μ g/ml propidium iodide (Nacalai Tesque). The cell cycle distribution was analyzed using Attune NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific K.K.) with FlowJo software (BD Biosciences, San Jose, CA).

TUNEL assay

Each cell line was seeded into 12-well plate at 2.0×10^5 cells/well. Doxycycline was added to each well at a concentration of 1 μ M. After 48h, the cells were fixed by 4% paraformaldehyde for 20 min, and followed by permeabilization using PBS-T (PBS

mixed with 0.5% Tween-20 and 0.2% bovine serum albumin) for 30min. Then the cells were incubated with FITC-dUTP and TdT from the Apoptosis TUNEL Kit Direct (Medical & Biological Laboratories Co., Ltd., Tokyo, Japan), according to the manufacturer's instructions. The samples were visualized at BZ-X800 (KEYENCE, Osaka, Japan). The percentages of TUNEL-positive cells were counted by the built-in optical hybrid cell count mode of BZ-X800.

Western blotting

Western blotting analysis was performed according to the previous report (Inanaga et al., 2021). Briefly, each cell line was harvested and lysed with NP40 lysis buffer (1% NP40, 10 mM Tris HCl (pH 7.5), 150 mM NaCl, and 1 mM EDTA). The protein lysates were electrophoresed in 13.5% acrylamide gel before transferring onto membranes. The membranes were incubated with each primary antibody (Table III-1) overnight at 4°C. HRP-conjugated anti-mouse IgG antibody (Merck Millipore, Darmstadt, Germany) or HRP-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch, Philadelphia, PA) was used as a secondary antibody. The membranes were soaked in Chemi-Lumi One Super (Nacalai Tesque, Inc.) to visualize the immunoreaction. Immunoreactive bands were quantified and analyzed with iBright1500

(Thermo Fisher Scientific K.K.). The membranes were reprobed with mouse monoclonal anti-beta actin antibody (Sigma-Aldrich Japan K.K., Tokyo, Japan) to verify equal loading of proteins in each lane.

Dual luciferase assay

Each cell line was seeded into 6-well plate at 4.0×10^5 cells/well. The pRL-SV40 (Promega Corp., Madison, WI) and pGL4.38 (Promega Corp.) plasmids were transfected into each cell line using Lipofectamin®3000 (Thermo Fisher Scientific K.K.). Twenty-four hours after transfection, the cells were collected and re-seeded into 96-well plate at 5,000 cells/well in the presence of 1 μ M doxycycline. After 24h of culture, the cells were lysed using the Dual-Glo Luciferase Assay System (Promega Corp.), and luciferase activity was measured using VARIOSKAN LUX (Thermo Fisher Scientific K.K.).

In prediction of protein structure and molecular interactions

To identify structural differences between p53 protein in African pygmy hedgehogs and humans, the three-dimensional (3D) structures of each were predicted using AlphaFold 3 (Google DeepMind, London, UK). For both species, structural models

were generated under two conditions—presence and absence of the p53 responsive elements—to examine conformational changes associated with DNA binding.

Furthermore, to assess potential interactions between p53 and SH3 domain-containing proteins, the predicted p53 structures of both species were analyzed using two tools: SCRIBER (Zhang and Kurgan, 2019) and cons-PPISP (Chen and Zhou, 2005; Qin and Zhou, 2007). These analyses enabled prediction of protein–protein interaction sites and comparative evaluation of binding propensities between the two species.

Statistical analysis

The ANOVA and Tukey–Kramer test determined the significance of differences between samples. Multiple comparison test was performed using JMP Pro 15 software (SAS Institute Japan, Tokyo, Japan). Statistical significance was set at $p < 0.05$.

RESULTS

Comparison of tumor-associated genes between African pygmy hedgehogs and humans

Because a complete genome sequence of African pygmy hedgehogs is not yet available, I obtained a draft genome from Project Kalunguyeye. Based on this draft genome, I annotated 17 tumor-associated genes frequently mutated in human cancers (Chang et al., 2017) (Table V-3). To identify genes that may contribute to tumorigenesis in this species, I compared the transcript sequences of these genes with their human orthologs. Among them, African pygmy hedgehogs-p53 (Afh-p53) showed remarkably lower sequence identity to human p53 (h-p53), as shown in Table V-3 and Figure V-1A. Notably, the transactivation domains (TAD1 and TAD2) and the proline-rich domain (PRD) of Afh-p53 exhibited particularly low conservation.

To investigate interspecies differences in greater detail, I performed a multiple sequence alignment and constructed a phylogenetic tree based on p53 amino acid sequences from 40 vertebrate species (Figure V-1B). Interestingly, Afh-p53 did not cluster with other members of the superorder Laurasiatheria, to which the African pygmy hedgehogs taxonomically belong. This unexpected phylogenetic separation suggests that Afh-p53 may retain functionally primitive features among mammals. Therefore, I focused on characterizing the functional properties of Afh-p53 in this study.

Establishment of Tet-On-inducible recombinant TP53 mutants

To establish cell lines expressing p53 mutants, I first screened for an anti-p53 monoclonal antibody capable of detecting Afh-p53. Based on partial conservation of the PAb240 epitope between humans and African pygmy hedgehogs (Stephen and Lane, 1992) (Figure V-2A), I selected the PAb240 clone for validation. I generated *TP53* gene knockout African pygmy hedgehogs cell lines using HHOVT cells and confirmed complete loss of endogenous Afh-p53 expression. Western blotting revealed that PAb240 cross-reacted with Afh-p53, as the signal was absent in *TP53*-knockout cells (Figure V-2B).

Next, *TP53*-null human cell lines—Saos-2 (osteosarcoma) and H1299 (non-small cell lung cancer)—were transduced with expression constructs encoding p53 mutants (Figure V-2C). Because constitutive expression of h-p53 induces cell death and cell cycle arrest in both Saos-2 and H1299 cells (Ganjavi et al., 2006; Liu et al., 2017), a Tet-On inducible expression system was used to enable cell maintenance (Figure V-2D). I generated six recombinant constructs: h-p53, Afh-p53, hhh-Afh-p53 (Afh-p53 carrying human TAD1, TAD2, and PRD), hha-Afh-p53 (human TAD1 and TAD2), and hah-Afh-p53 (human TAD1 and PRD).

Evaluation of tumor suppressive activities of each p53 protein

To investigate the function of Afh-p53, I performed cell proliferation and cytotoxicity assays in Saos-2 and H1299 cell lines expressing each p53 mutants (Figure V-3A and 3B). Consistent with previous reports (Schuler et al., 2000; Chen et al., 2019), h-p53 robustly inhibited cell proliferation and induced cell death. In contrast, Afh-p53 induced only moderate growth inhibition and cytotoxicity. Notably, hhh-Afh-p53 induced cell death and inhibited cell proliferation comparable to that of h-p53, whereas hha-Afh-p53 behaved similarly to Afh-p53. These findings indicate that reduced function of Afh-p53 may be attributable to differences within its TADs and/or PRD.

To confirm these results in African pygmy hedgehogs-derived cell lines, *TP53* knockout-HHOVT cells were transduced with each p53 expression construct (Figure V-3C). Because the parental HHOVT cell line is p53-wild type, I anticipated that p53 expression alone would not efficiently induce apoptosis. Thus, to assess p53-dependent anti-tumor activity, I compared the sensitivity of each cell line to doxorubicin treatment. HHOVT cells expressing h-p53 were more sensitive to doxorubicin than those expressing Afh-p53 (Figure V-3D), indicating that Afh-p53 elicits a weaker apoptotic response to DNA damage.

Afh-p53 induces weaker apoptosis than h-p53

To investigate the mechanism underlying reduced cytotoxicity, I first analyzed the cell cycle distribution of the cells expressing each p53 mutant. Flow cytometric cell cycle analysis revealed that all p53-expressing cells underwent comparable G0/G1 arrest (Figure V-4A), consistent with similar expression levels of p21, a p53 target protein involved in cell cycle regulation (Figure V-4D).

Next, I examined the apoptotic induction by each p53 using a TUNEL assay. In agreement with the results of the cytotoxicity assay, cells expressing Afh-p53- or hha-Afh-p53 exhibited markedly fewer TUNEL-positive cells than those expressing h-p53 or hhh-Afh-p53 (Figure V-4B and 4C). These cells also exhibited reduced caspase-3 cleavage (Figure V-4D), indicating attenuated activation of the apoptotic pathway. Collectively, these findings demonstrate that although Afh-p53 retains the ability to induce cell cycle arrest, its pro-apoptotic activity is substantially impaired relative to h-p53.

Afh-p53 exhibits reduced transactivation of pro-apoptotic genes

To elucidate the molecular basis of reduced apoptosis, I performed western blot analysis of p53 target proteins involved in apoptotic signaling. Expression of Puma and Noxa was substantially lower in Aft-p53- and hha-Afh-p53-expressing cells than in h-p53-expressing cells. BAX expression—regulated directly by p53 and indirectly via Puma and Noxa—was likewise decreased (Figure V-5A).

Because these p53 target genes contain p53-responsive elements in their promoters, I evaluated the transcriptional activity of each p53 mutant using a luciferase reporter containing a p53 responsive elements. Consistent with the protein expression data, Afh-p53 and hha-Afh-p53 exhibited reduced transactivation capacity compared with h-p53 (Figure V-5B).

Structural and molecular modeling of attenuated apoptotic activity in Afh-p53

The reduced induction of apoptotic genes by Afh-p53 raised the possibility that Afh-p53 might simply be unable to bind human p53 responsive elements due to interspecies sequence differences. To address this possibility, I predicted 3D structures of each p53 mutant and simulated their interactions with human p53 responsive elements using AlphaFold 3. All p53 mutants exhibited comparable predicted binding to human

p53 responsive elements, and no major structural differences were observed (data not shown), likely because the TAD-PRD region is intrinsically disordered.

Because Afh-p53 and hha-Afh-p53 consistently showed similar phenotypes, I hypothesized that the PRD of Afh-p53 contributes to its reduced apoptotic function. The PRD contains multiple PXXP motifs that serve as binding sites for Src-homology 3 (SH3) domain-containing proteins such as ASPP1 (Yu et al., 1994), which enhances transcription of pro-apoptotic genes by interacting with p53 (Bergamaschi et al., 2004). To investigate whether impaired PRD-mediated interactions underlie the reduced function of Afh-p53, I performed molecular docking simulations to assess binding between each PRD and SH3 domains (Figure V-6). Afh-p53 exhibits reduced predicted affinity, consistent with its fewer PXXP motifs. These findings suggest that diminished binding to ASPP1 and other SH3-domain proteins contributes to the attenuated apoptotic activity of Afh-p53.

DISCUSSION

In this study, I demonstrated that *TP53* gene of the African pygmy hedgehogs (*Atelerix albiventris*) may retain more “ancestral-like” functional features compared with those of other placental mammals, based on draft genome analysis and comparative sequence data. Functional analyses further revealed that Afh-p53 exhibited a reduced capacity to induce apoptosis relative to h-p53, both *in vitro* and *silico*. These findings provide molecular insight that may partly account for the high tumor incidence observed in this species despite its small body size.

While research on Peto’s paradox has traditionally focused on large and/or long-lived animals to elucidate mechanisms of enhanced tumor suppression, studies addressing tumorigenesis in small and/or short-lived species remain limited. The present results highlight a complementary perspective by examining intrinsic tumor-risk factors in the African pygmy hedgehogs. Importantly, the *TP53* gene of this species should not simply be regarded as “weakened” relative to that of the other species. A global reduction in tumor-suppressive capacity would be expected to impose substantial fitness cost, making an evolutionary shift toward tumor susceptibility unlikely. Rather, the present data are consistent with the possibility that *TP53* of African pygmy hedgehogs retains a more ancestral-like functional state, whereas other lineages—including humans—have

progressively strengthened their tumor-suppressive mechanisms during evolution. This perspective underscores that a full understanding of Peto's paradox requires not only studying the enhanced tumor suppression in large, long-lived animals but also clarifying the tumor-control strategies employed by more typical-sized mammals.

The propensity to undergo apoptosis in response to genotoxic stress differs among species (Caulin and Maley, 2011). For example, a previous study showed that mouse cells are more resistant to DNA damage-induced apoptosis than human cells (Humbert et al., 1999). Another study reported no correlation between tumor incidence and the strength of the DNA damage response to UV and doxorubicin among fibroblasts from multiple species (Compton et al., 2025); in that work, fibroblasts derived from African pygmy hedgehogs exhibited a markedly weaker response than fibroblasts from ferrets, which show a tumor incidence of 63%. Although this assay does not isolate any single molecular pathway, the diminished response in African pygmy hedgehog fibroblasts is broadly consistent with the reduced apoptotic capacity of Aflh-p53 observed in the present study. Nevertheless, it should be noted that these data were obtained from tumor-derived hedgehog cells, which may not fully reflect normal physiological conditions.

In this study, I found that the reduced apoptotic capacity of Aflh-p53 may be

partly attributable to the decreased number of PXXP motifs within its PRD. The PRD of p53 has been implicated in stabilization of p53 and in the promotion of p53-dependent transcription, for example by inhibiting MDM2-dependent degradation via binding to Pin1 and by facilitating interactions with reinforcement transcriptional co-factors (Berger et al., 2001, 2005; Zheng et al., 2002; Mantovani et al., 2004). Previous reports have described loss of both cell cycle arrest and apoptosis upon disruption of the PRD (Baran et al., 2017). However, there is considerable inconsistency regarding PRD function. Some studies have reported a reduction only in growth inhibition when the PRD is absent (Walker and Levine, 1996; Toledo et al., 2006a), whereas others have shown that PRD loss preferentially impairs apoptosis while leaving cell cycle arrest relatively intact (Sakamuro et al., 1997; Roth et al., 2000; Baptiste et al., 2002; Slatter et al., 2009). The latter findings are in line with the present results showing that Afh-p53 retains the ability to induce cell cycle arrest but exhibits attenuated apoptotic activity. Taken together, these observations raise the possibility that the PRD may act as a context-dependent “switch” that modulates the balance between apoptosis and cell cycle arrest in response to intra- and extracellular conditions. Additionally, Toledo, et al. showed that interaction of the PRD with Pin1 stabilizes p53 without markedly enhancing apoptosis (Toledo et al., 2006b). I also identified putative Pin1-binding sites within Afh-p53 (data not shown),

suggesting that Pin1-mediated stabilization is likely preserved in this species. In contrast, several studies have argued that the PXXP motif per se does not critically contribute to protein interactions within the PRD, instead proposing a functional role for the PXXXXP motifs and suggesting that the PRD may serve as a structural spacer (D'Erchia et al., 1999; Toledo et al., 2006b). Although the present results in which a reduced number of PXXP motifs correlates with diminished apoptotic function, appear to differ from these reports, the substantial interspecies diversity in the PRD implies that multiple structural features, not limited to the PXXP motif, may cooperatively shape p53 function. Furthermore, while spontaneous thymic lymphomas increase in p53^{-/-} mice, PRD-deficient mice exhibit resistance to tumor development and prolonged survival even after γ -irradiation (Toledo et al., 2006a; Adams et al., 2015), although a tendency toward late-onset B cell lymphomas has also been reported (Slatter et al., 2009). These findings collectively suggest that the high tumor incidence observed in African pygmy hedgehogs cannot be explained solely by the properties of Afh-p53 and that additional contributing factors must be considered.

Multiple life-history and ecological traits—including litter size, placental invasiveness, gestation length, social structure, and diet—has been proposed as potential modifiers of tumor risk across species (D'Souza and Wagner, 2014; Boddy et al., 2020;

Vincze et al., 2022; Wagner et al., 2022; Dighe et al., 2024; Sierra et al., 2025), but their associations with tumorigenesis are inconsistent. This discrepancy is likely influenced by other variables such as body size, lifespan, and reproductive strategies. The African pygmy hedgehog possesses a hemochorial placenta, 1 to 9 offspring per litter, and has an omnivorous diet that includes both invertebrates and small vertebrates (Ferner et al., 2014). In addition, retroviral particles have been detected in tumor tissues of African pygmy hedgehogs, although their relationship to tumorigenesis remains unclear (Peauroi et al., 1994; Raymond et al., 1998). These endogenous and exogenous factors may collectively contribute to tumor susceptibility in this species, in conjunction with the attenuated apoptotic function of Afh-p53.

I was unable to directly assess tumor incidence in the closest relative of the African pygmy hedgehog, the Western European hedgehog (*Erinaceus europaeus*), whose genome was used for gene annotation. Several epidemiological reports have documented tumor incidences of approximately 4-6% in wild Western European hedgehogs (Rautio et al., 2016; Garcês et al., 2020; Zacharopoulou et al., 2022). Although these data are subject to biases inherent to wildlife rescue populations, such as trauma and nutritional imbalances, they suggest that neoplastic disease may also be relatively common in this closely related species.

A key limitation of this study is that apoptosis-related genes such as *Puma* and *Noxa* could not be identified in the African pygmy hedgehog genome during the present gene annotation process. If *TP53* in this species represents a more ancestral-like state, these genes may be absent, highly diverged, or otherwise difficult to detect with current resources. It is also conceivable that additional, uncharacterized components of apoptotic pathways exist in this species. As a result, detailed mechanistic analyses using African pygmy hedgehog-cell lines were challenging. Future studies employing primary hedgehog tissues and species-specific genomic resources will be essential to determine whether similar molecular phenomena occur under more physiological conditions.

In conclusion, the present results suggest that functional differences in the *TP53* gene, particularly within its PRD, may represent one of the molecular mechanisms contributing to the unusually high incidence of neoplasia in the African pygmy hedgehog. Taken together, this study advances the understanding of the molecular basis of tumor susceptibility in small mammals and provides novel insight into the evolutionary diversity of tumor-suppressive strategies across species.

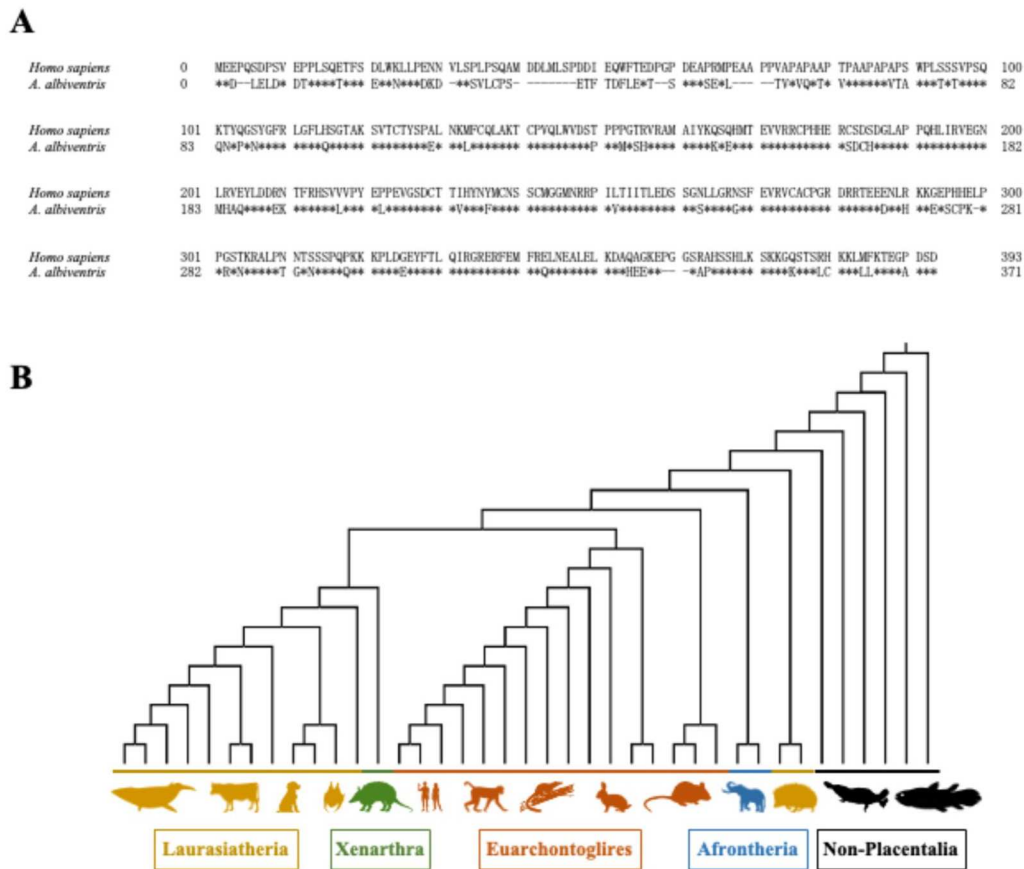


Figure V-1. Alignment of the p53 amino acid sequence of *Atelerix albiventris*.

(A) Multiple sequence alignment of p53 proteins among indicated species. An asterisk indicates an amino acid identical to that of human. A dash indicates the absence of an amino acid. (B) Phylogenetic tree constructed from the multiple sequence alignment of p53 proteins from 40 vertebrates. The animals shown as silhouettes represent typical species within each taxonomic group and color-filled according to their superorders (Afrotheria, Euarchontoglires, Xenarthra, and Laurasiatheria).

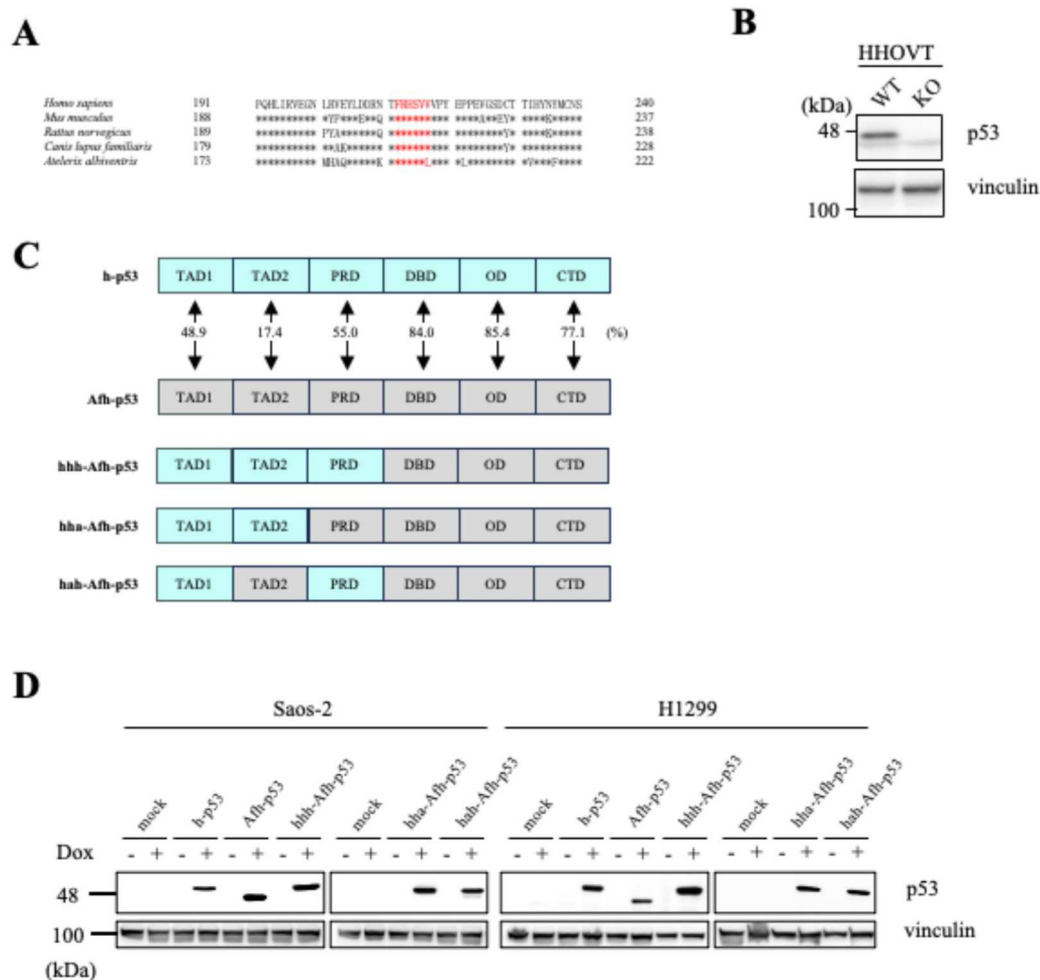


Figure V-2. Establishment of cell lines expressing p53 constructs.

(A) Alignment of partial p53 amino acid sequences. The epitope recognized by the anti-p53 monoclonal antibody PAb240 is highlighted in red. (B) Validation of cross-reactivity of an anti-human p53 antibody. Proteins extracted from WT and p53-knockout HHOVT cells were separated with SDS-PAGE, and analyzed by western blotting. (C) Schematic representations of human-p53 (h-p53), African pygmy hedgehogs-p53 (Afh-p53), and chimeric mutants. The percentage identify of each domain between human and African

pygmy hedgehog is indicated. TAD, Transactivation domain; PRD, Proline-rich domain; DBD, DNA binding domain; OD, Oligomerization domain; CTD, C-terminal domain.

(D) Inducible expression of each p53 construct in Saos-2 and H1299 cells. Cells were treated with 1 $\mu\text{g/ml}$ of doxycycline for 24h, followed by western blotting of p53 and vinculin as a loading control.

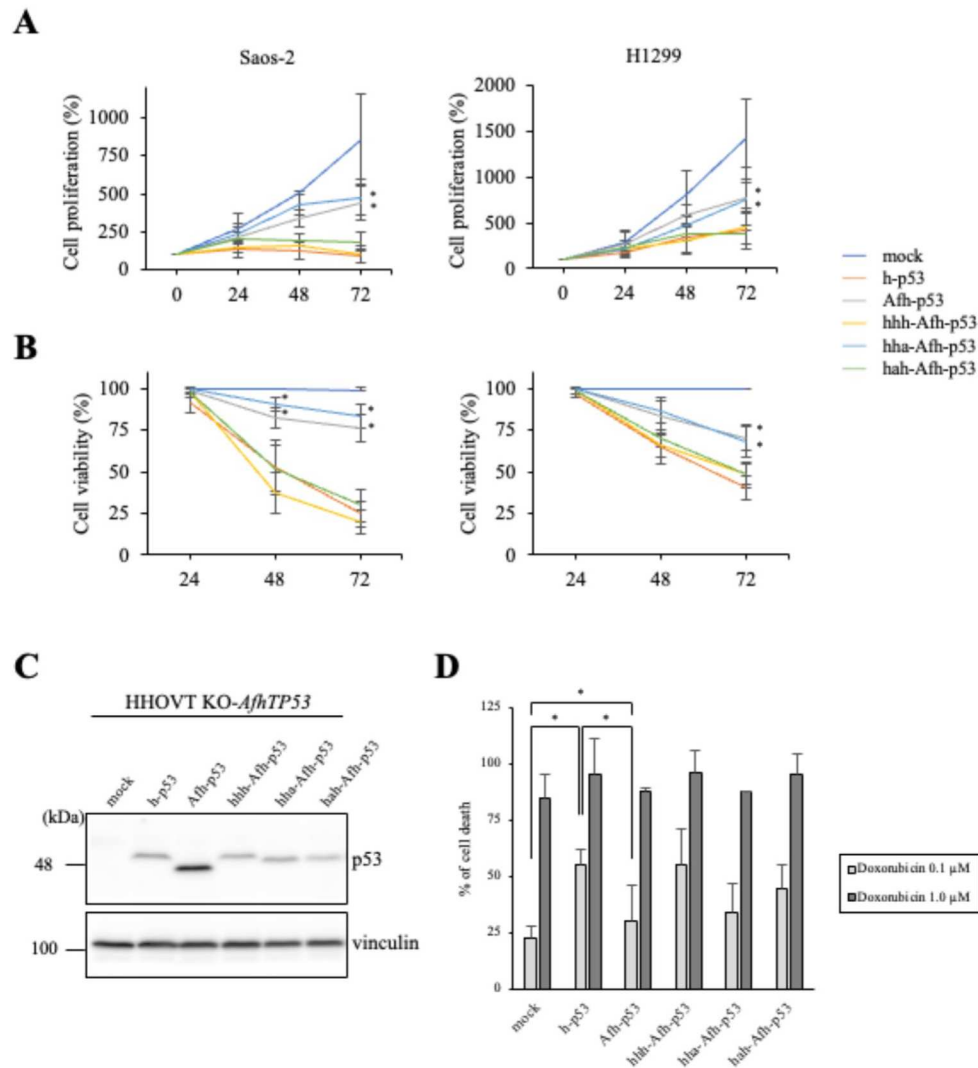


Figure V-3. Tumor-suppressive function of Aflh-p53 is attenuated compared with h-p53.

(A) Cell proliferation assay in Saos-2 and H1299 cells expressing each p53 construct. Cells were treated with 1 μ g/ml of doxycycline for 0 to 72h, and cell proliferation rate was quantified using Cell Counting Kit-8. The y-axis represents the proliferation ratio relative to 0 h (set to 1) at each time point. Data are presented as mean $\pm$ SD of three

independent experiments. Tukey-Kramer test. *, $P<0.05$. (B) Cytotoxicity assay. Cells treated with 1 $\mu\text{g/ml}$ of doxycycline for 24 to 72h were assessed for viability using trypan blue exclusion test. The y-axis represents the cell viability at each time point. The results were expressed as mean $\pm$ SD of three independent experiments. Tukey-Kramer test. *, $P<0.05$. (C) Establishment of HHOVT cell lines expressing each p53 construct. Cells were treated with 1 $\mu\text{g/ml}$ of doxycycline for 24h to induce the expression of p53. (D) HHOVT cells were treated with doxorubicin at the indicated concentrations for 48h. Cell death was evaluated by trypan blue exclusion. The y-axis indicates the percentage of dead cells. Data are shown as mean $\pm$ SD of three independent experiments. Tukey-Kramer test. *, $P<0.05$.

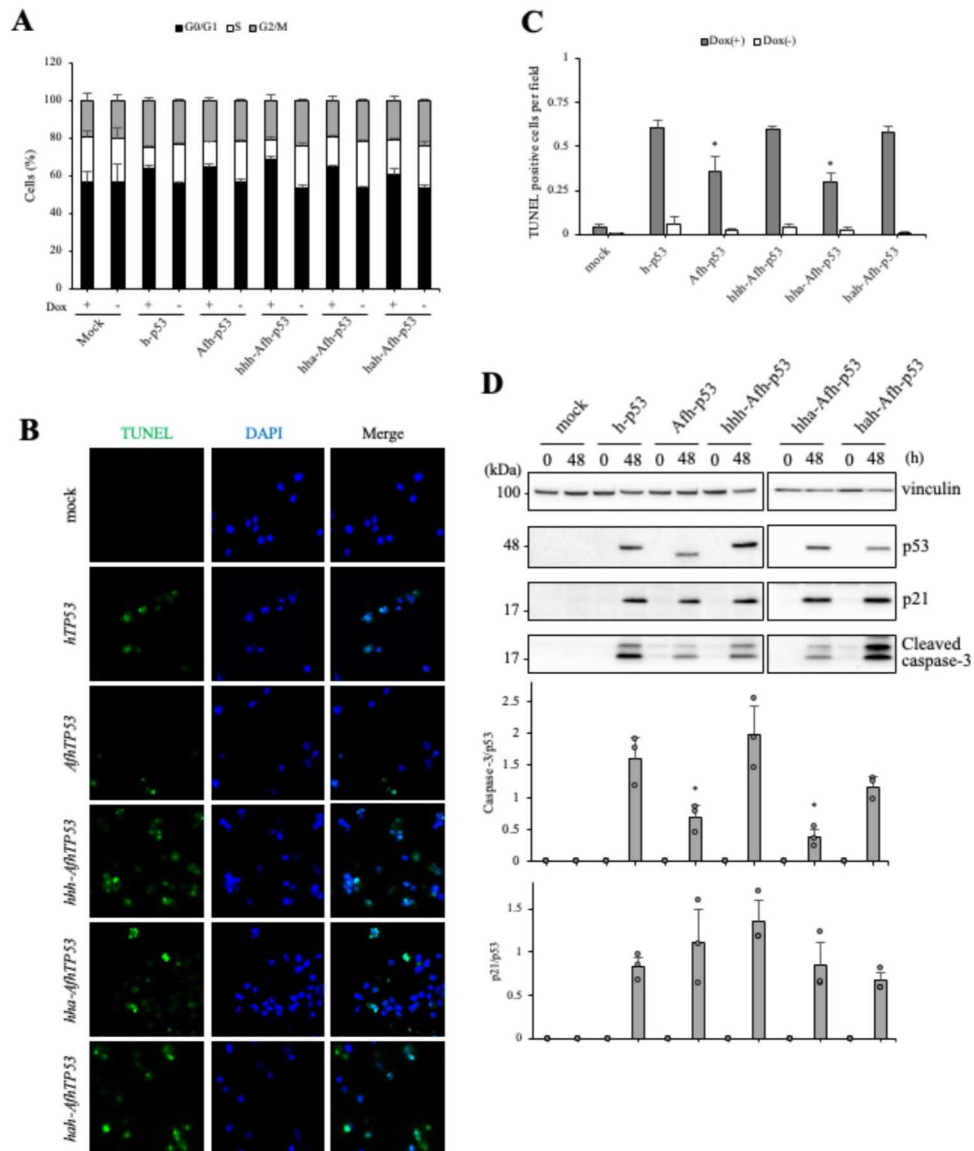


Figure V-4. Afh-p53 maintains cell cycle regulation but fails to induce apoptosis.

(A) Cell cycle analysis. The percentage of cells in the G0/G1, S, and G2/M phases after 48 h of doxycycline treatment are shown. The results were expressed as mean $\pm$ SD of three independent experiments. (B) Representative images of TUNEL staining (green) and DAPI (blue) after 48h of doxycycline treatment. (C) Quantification of TUNEL-

positive cells using the hybrid cell count mode of the BZ-X800 microscope. Data are mean $\pm$ SD. Tukey-Kramer test. *, $P<0.05$. (D) Western blot analysis of p21 expression and caspase-3 cleavage after 48 h of doxycycline treatment. Each blot represents one of three experiments showing a similar trend. The graphs show the expression levels of each protein normalized to p53, quantified using ImageJ software. Data represent mean $\pm$ SD of three independent experiments. Tukey-Kramer test. *, $P<0.05$.

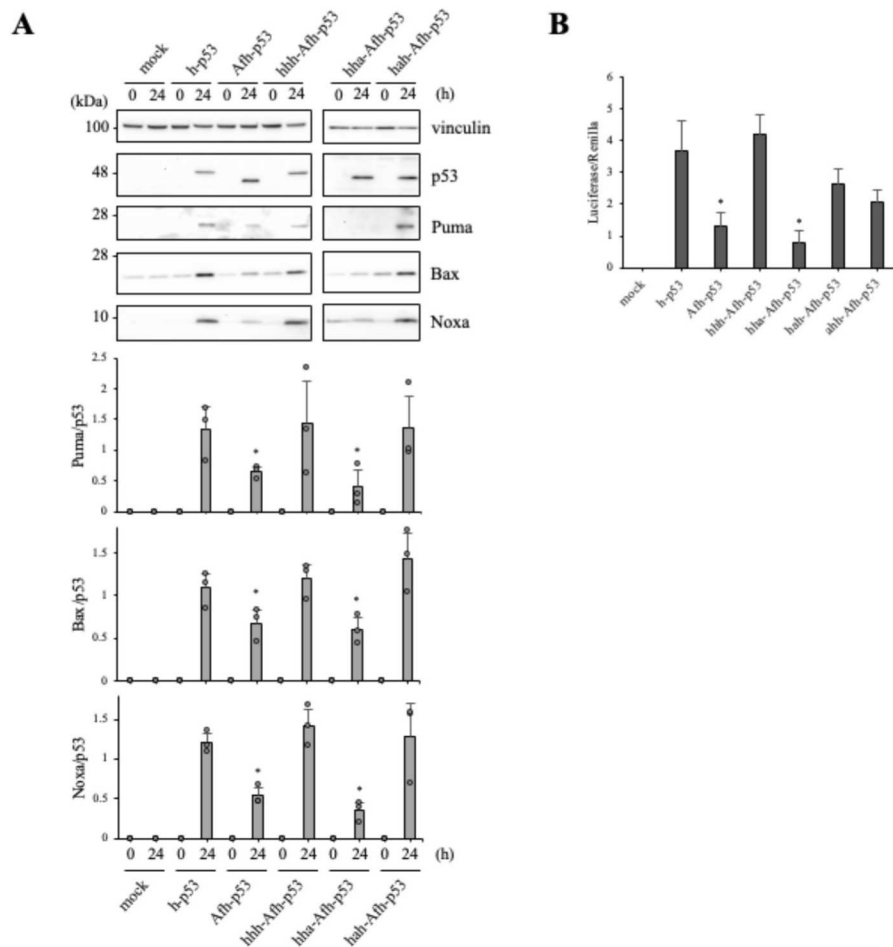


Figure V-5. Aflh-p53 shows reduced activation of downstream apoptotic pathways due to alterations in its proline-rich domain.

(A) Western blot analysis of pro-apoptotic proteins (Puma, Bax, and Noxa). Cells were treated with 1 μ g/ml of doxycycline for 24h. Each blot represents one of three experiments showing a similar trend. Graphs represent protein expression level normalized to p53 quantified using ImageJ software. The results were expressed as mean $\pm$ SD of three independent experiments. Tukey-Kramer test. *, $P < 0.05$ vs other mutants

except for hhh-Afh-p53 in the graphs of Puma. (B) Transactivation analysis of each p53 construct. Cells were transfected with a luciferase reporter containing a p53- responsive element and renilla luciferase served as a transfection control. The y-axis indicates the luminescence ratio of firefly luciferase to Renilla. The results were expressed as mean $\pm$ SD of three independent experiments. Tukey-Kramer test. *, $P < 0.05$.

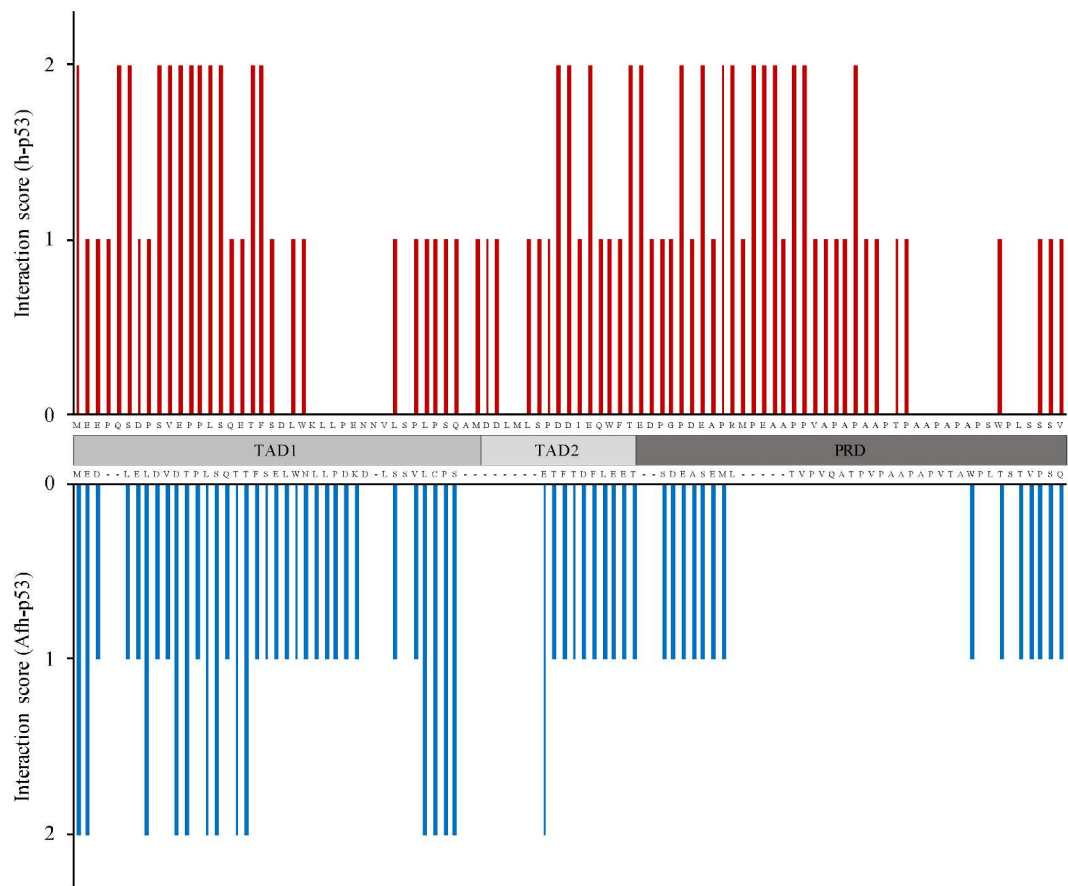


Figure V-6. *In silico* modeling suggests impaired interactions of Afh-p53 with binding partners.

Molecular docking simulations were performed using SCRIBER and cons-PPISP. The upper and lower graphs show the results of h-p53 and Afh-p53, respectively. The y-axis indicates the Interaction scores predicted by both models, and the x-axis indicates the amino acid sequences of each p53 that correspond to each other. Each domain is shown between the sequences. TAD; transactivation domain, PRD; proline-rich domain.

Table V-1. List of vertebrates used for multiple sequence alignment.

Common name	Scientific name	Genome assembly
African clawed frog	<i>Xenopus laevis</i>	Xenopus_laevis_v10.1
African elephant	<i>Loxodonta africana</i>	Loxafr3.0
African pygmy hedgehog	<i>Atelerix albiventris</i>	
Alpaca	<i>Vicugna pacos</i>	VicPac1
Armadillo	<i>Dasypus novemcinctus</i>	Dasnov3.0
Baboon	<i>Papio anubis</i>	Panubis1.0
Beluga	<i>Delphinapterus leucas</i>	ASM228892v3
Cat	<i>Felis silvestris catus</i>	F.catus_Fca126_mat1.0
Cattle	<i>Bos taurus</i>	ARS-UCD2.0
Chicken	<i>Gallus gallus</i>	bGalGal1.mat.broiler.GRCg7b
Chimpanzee	<i>Pan troglodytes</i>	Pan_tro_3.0
Coelacanth	<i>Latimeria chalumnae</i>	LatCha1
Dog	<i>Canis lupus familiaris</i>	ROS_Cfam_1.0
Ferret	<i>Mustela putorius furo</i>	MusPutFur1.0
Gibbon	<i>Nomascus leucogenys</i>	Nleu_3.0
Golden Hamster	<i>Mesocricetus auratus</i>	MesAur1.0
Gorilla	<i>Gorilla gorilla gorilla</i>	gorGor4
Greater horseshoe bat	<i>Rhinolophus ferrumequinum</i>	mRhiFer1_v1.p
Guinea pig	<i>Cavia porcellus</i>	Cavpor3.0
Horse	<i>Equus caballus</i>	EquCab3.0
Human	<i>Homo sapiens</i>	GRCh38.p14
Hyrax	<i>Procavia capensis</i>	proCap1
Marmoset	<i>Callithrix jacchus</i>	mCalJac1.pat.X
minke whale	<i>Balaenoptera acutorostrata</i>	mBalAcu1.1
Mouse	<i>Mus musculus</i>	GRCm39
Mouse Lemur	<i>Microcebus murinus</i>	Mmur_3.0
Naked mole-rat	<i>Heterocephalus glaber</i>	Naked_mole-rat_maternal
Orangutan	<i>Pongo abelii</i>	Susie_PABv2
Painted turtle	<i>Chrysemys picta</i>	Chysemys_picta_bellii-3.0.3
Pig	<i>Sus scrofa</i>	Sscrofa11.1
Platypus	<i>Ornithorhynchus anatinus</i>	mOrnAna1.p.v1
Rabbit	<i>Oryctolagus cuniculus</i>	OryCun2.0
Rat	<i>Rattus norvegicus</i>	GRCr8
Rhinoceros	<i>Diceros bicornis</i>	mDicBic1.mat.cur
Sheep	<i>Ovis aries</i>	ARS-UI_Ramb_v2.0
Sperm whale	<i>Physeter macrocephalus</i>	ASM283717v5
Tarsier	<i>Tarsius syrichta</i>	Tarsius_syrichta-2.0.1
Tasmanian devil	<i>Sarcophilus harrisii</i>	mSarHar1.11
Tree shrew	<i>Tupaia belangeri</i>	tupBel1
Western European hedgehog	<i>Erinaceus europaeus</i>	eriEur1

Table V-2. Primary antibodies used in this study.

Antibody	Clone	Type	Company	Dilution	Use for
p53	PAb240	Mouse monoclonal	Santa Cruz Biotechnology	1:1000	Western blotting
p21	C-19	Rabbit monoclonal	Santa Cruz Biotechnology	1:1000	Western blotting
Bax	2D2	Mouse monoclonal	Santa Cruz Biotechnology	1:1000	Western blotting
Puma	G-3	Mouse monoclonal	Santa Cruz Biotechnology	1:1000	Western blotting
Noxa	114C307	Mouse monoclonal	Santa Cruz Biotechnology	1:1000	Western blotting
cleaved caspase-3	5A1E	Rabbit monoclonal	Cell Signaling Technology	1:1000	Western blotting
vinculin	7F9	Mouse monoclonal	Santa Cruz Biotechnology	1:1000	Western blotting

Table V-3. Identification of amino acid sequences in *A. albiventris* compared to human's.

Gene	Homology (%)
<i>AKT1</i>	95.3
<i>APC</i>	97.9
<i>BRAF</i>	97.9
<i>CTNNB1</i>	94.3
<i>EGFR</i>	80.2
<i>ERBB2/HER2</i>	85.9
<i>ERBB3</i>	89.7
<i>FBXW7</i>	94.1
<i>GNAQ</i>	99.7
<i>HRAS</i>	98.2
<i>IDH1</i>	93.8
<i>IDH2</i>	94.2
<i>KRAS</i>	98.4
<i>PIK3CA</i>	97.5
<i>RAC1</i>	92.4
<i>SMAD4</i>	95.1
<i>TP53</i>	67.0

GENERAL DISCUSSION

In Chapter 1, I investigated the expression of mismatch repair (MMR) proteins in canine tumor tissues by immunohistochemistry. Loss of MMR proteins expression was observed in a variety of tumor types, particularly in oral malignant melanomas and hepatocellular carcinomas. These findings indicate that mismatch repair deficiency (dMMR) occurs in canine tumors, similar to the situation in human cancers. Notably, canine oral malignant melanomas have been reported to exhibit relatively high responsiveness to immune checkpoint inhibitors (ICIs) (Igase et al., 2020c, 2022). The presence of dMMR may partly account for this clinical response. Taken together, these observations suggest that dMMR is a potential biomarker candidate for predicting the efficacy of ICIs in canine tumors.

In Chapter 2, I examined the prevalence of microsatellite instability (MSI) in canine tumors. MSI was frequently detected in multiple tumors types, particularly in oral malignant melanomas, which is consistent with the high frequency of dMMR observed in Chapter 1. Furthermore, cases with dMMR showed a significantly higher MSI frequency than those with MMR proficiency, supporting the relationship between MSI and dMMR in canine tumors. In humans, both dMMR and MSI are established as tumor type-agnostic biomarkers for predicting ICIs efficacy. To establish dMMR and MSI as reliable biomarkers in canine oncology, further studies are required to determine their

utility in predicting treatment responses to ICIs in dogs. In addition, careful selection and optimization of microsatellite marker sets will be needed to improve the sensitivity and specificity of MSI detection. An additional contribution of Chapter 1 and 2 is the establishment of practical methods for detecting dMMR and MSI in canine tumors. These procedures provide a methodological foundation for future research on immunotherapies and on hereditary cancer syndromes in veterinary medicine.

Next, in Chapter 3, I demonstrated that canine KIT exon 11 mutants are aberrantly localized to the endoplasmic reticulum (ER), where they constitutively activate STAT5 signaling. This finding provides new insight into the intracellular behavior of mutant KIT, highlighting that oncogenic signaling can be initiated before KIT reaches the plasma membrane. Moreover, this chapter suggested an association between the *c-kit* exon 11 mutations and specific KIT immunostaining patterns. In particular, ER accumulation of mutant KIT offers a mechanistic explanation for the poor clinical outcomes observed in canine mast cell tumors and gastrointestinal stromal tumors exhibiting KIT pattern III. Collectively, these findings raise the possibility that ER-localized KIT itself may represent a therapeutic target. Targeting the aberrant intracellular trafficking or ER-associated signaling of KIT could provide a novel treatment strategy that is distinct from conventional tyrosine kinase inhibitors and other anti-tumor drugs.

To translate this concept into clinical applications, further studies are needed to elucidate the detailed molecular mechanisms underlying ER retention, evaluate the druggability of ER-localized KIT signaling, and explore therapeutic strategies aimed at modulating the subcellular localization of KIT.

Finally, I focused on tumorigenesis in the Africa pygmy hedgehogs (*Atelerix albiventris*). Although this species is known as an exotic companion animal with a high incidence of tumors, research resources for this species have been very limited. It was therefore necessary to establish a tumor cell line and to obtain genomic sequence information as fundamental tools for further research.

In Chapter 4, I established a histiocytic sarcoma (HS) cell line, HHMYT, from an African pygmy hedgehog. Using this cell line, I demonstrated the anti-tumor effects of the proteasome inhibitors bortezomib, carfilzomib, and disulfiram through drug screening assay, and further analyzed their anti-tumor mechanisms of action in HHMYT cells. These findings indicated that proteasome inhibitors may represent promising therapeutic candidates for HS in African pygmy hedgehogs. Future studies are required to evaluate their clinical efficacy against HS and to characterize the pharmacokinetics and safety profiles of these drugs in this species.

In Chapter 5, to identify endogenous factors that may underlie the unusually

high tumor incidence in African pygmy hedgehogs, I analyzed a draft genome assembly of this species. I identified marked structural divergence in its *TP53* gene, particularly within the transactivation domain and proline-rich domain (PRD). Functional assays using recombinant p53 constructs demonstrated that African pygmy hedgehogs-p53 (Afh-p53) induced significantly weaker apoptosis than human-p53 in both human and hedgehog-derived cell lines, owing to differences in its PRD. These results suggest that functional alterations in the *TP53* gene, particularly within its PRD, may represent one of the molecular mechanisms contributing to the unusually high incidence of neoplasia in the African pygmy hedgehog.

Taken together, my experiments provide both fundamental and clinicopathological insights into neoplastic disease in dogs and African pygmy hedgehogs. Research on companion animals other than dogs and cats remains limited in veterinary medicine, and these studies presented in this thesis have further deepened my interest in comparative oncology that encompasses a broader diversity of species. I hope that the findings from this work will contribute to improved clinical outcomes through future translational clinical applications and will stimulate the discovery of new knowledge in veterinary and comparative oncology.

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