博士論文（要約）

Developing and optimizing chimeric antigen receptor T cell approaches for the treatment of canine cancers

（イヌの悪性腫瘍に対する キメラ抗原受容体発現 T 細胞療法の開発と最適化）

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Contents

List of Abbreviation

ACT	Adoptive cell transfer
ALL	Acute lymphoblastic leukemia
APC	Antigen presenting cell
ASAC	Anal sac adenocarcinoma
BCL	B cell lymphoma
CAFs	Cancer-associated fibroblasts
CAR	Chimeric antigen receptor
CasMab	Cancer-specific monoclonal antibody
CD	Cluster of differentiation
CTLA-4	Cytotoxic T-lymphocyte-associated protein-4
DC	Dendritic cells
DPBS	Dulbecco's phosphate-buffered saline
EGFR	Epidermal growth factor receptor
FACS	Fluorescence-activated cell sorting
FDA	Food and Drug Administration
FSC	Forward scatter
GFP	Green fluorescent protein
HCC	Hepatocellular carcinoma
HER2	Human epidermal growth factor receptor 2
HRP	Horseradish peroxidase
HSA	Hemangiosarcoma
ICS	Intracellular cytokine staining
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
ION	Ionomycin
LAK	Lymphokine activated killer
MAC	Mammary adenocarcinoma
MFI	Median fluorescence intensity
MGT	Mammary gland tumor
MHC	Major histocompatibility
MM	Malignant melanoma
OSA	Osteosarcoma
PAC	Pulmonary adenocarcinoma

PBL	Peripheral blood lymphocyte
PCR	Polymerase chain reaction
PD-1	Programmed cell death-1
PD-L1	Programmed cell death-ligand 1
PDPN	Podoplanin
PLC	Primary lung cancer
PMA	Phorbol 12-myristate 13-acetate
RCR/L	Replication-competent retrovirus/lentivirus
SCC	Side scatter
scFv	Single-chain variable fragment
SD	Standard deviation
SMA	Smooth muscle actin
SSC	Squamous cell carcinoma
TBS	Tris-buffered saline
TC	Thyroid cancer
TCC	Transitional cell carcinoma
TCM	T cell media
TCR	T cell receptor
THY	Thymoma
TIL	Tumor infiltrating lymphocyte
TNF	Tumor necrosis factor

General introduction

Prevalence of cancer and current therapeutic options in humans and dogs

Cancer is the one of the most common causes of disease-related death in humans. According to a report from the Global Cancer Observatory, there was an estimated 18.1 million new cancer cases and 9.6 million cancer deaths globally in 2018[1,2]. The National Cancer Institute also reports cancer statistics facts in the Surveillance, Epidemiology, and End Results Program[3]. In the report, approximately 39.5% of people in the United States were diagnosed with some form of cancer during their lifetime, based on data collected between 2015 and 2017, and for 2020, the estimated number of new cases of cancer and cancer related deaths were approximately 1.8 and 0.6 million respectively. It was also reported that approximately one third of cancer patients in the US do not survive longer than five years.

The morbidity and mortality of cancer is also high in dogs. A necropsy survey reported nearly half of dogs aged 10 years or older died of cancer[4]. Although the true incidence or prevalence of canine cancer is currently not known because of limited numbers of reports, estimates of canine cancer incidence range from 99.3 to 804 per 100,000 dogs in a year[5–7], which is comparable to the number of human cancer patients diagnosed (442.4 per 100,000 people per year between 2013-2017). The wide variation in cancer incidence in dogs amongst these surveys may be due in part to differences in

study methods, location and other variables, but the timing of the surveys probably affects the variation, since the prevalence of cancer has risen significantly in the past half century.

Because of increasing morbidity and high mortality of cancer, there is an increasing demand for effective treatments for human and canine cancer patients. Although the types of treatment that cancer patients receive depends on the type and stage of cancer, cancer treatments in general comprise three main modalities; surgery, chemotherapy and radiation therapy. However, the efficacy of these therapies either alone or in combination are oftentimes limited especially for patients with late stage cancer or cancer that is chemo- or radiation resistant. Thus, novel strategies are desperately required to treat cancer patients more effectively, and various alternative approaches including targeted small molecule inhibitors, hormone therapy and stem cell transplant for the treatment of cancer patients have been developed and are now routinely employed.

Cancer immunotherapy for the treatment of cancer patients

Immunotherapy approaches in cancer treatment have rapidly developed over the past decade, and immunotherapy is now the fourth pillar of cancer therapy in human medicine. The basic premise of cancer immunotherapy is to promote the host's immune response against cancer cells leading to their elimination from their body.

The immune system is well known as a crucial system to eliminate various kinds of pathogens such as bacteria, virus and parasites. However, the immune system also has an important role in detecting and eliminating cancer. Over the past few decades, the mechanisms by which the immune system detects and eliminates cancer have been more clearly understood. Chen *et al.* proposed the cancer immunity-cycle composed of 7 serial steps initiated by recognizing cancer cell antigens created through oncogenesis (step 1) (Fig. A)[8]. Antigen presenting cell (APC) (e.g. dendritic cells (DC)) take up and process neoantigens and present the captured antigen on major histocompatibility class (MHC) molecules to T cells (step 2), leading to priming and activation of effector T cell responses (step 3). Then, the activated effector T cells traffic to the tumor site (step 4) and infiltrate the tumor tissue (step 5). Finally, T cells recognize via their cognate peptides bound to MHC class I via their T cell receptor (TCR) (step 6) and kill cancer cells (step 7). Killing of the cancer cells releases additional tumor-associated antigens,

leading to increase the breadth and depth of the response in subsequent revolutions of the cancer immunity-cycle. Thus, host organisms have the ability to eliminate cancer, but this cancer immune cycle does not work properly in cancer patients. In some cases, tumor antigens were not recognized optimally due to lack of MHC on the tumor cells. In other cases, DCs and T cells treat antigens as self rather than foreign thereby creating regulatory T cells (Treg) response rather than effector responses, leading to suppress cancer immunity-cycle. In other cases, T cells do not properly home to tumors or kill tumor cells because of immune suppression by tumor microenvironment, such as release of immunosuppressive factors and T cell checkpoint dysregulation.

The goals of cancer immunotherapy are to initiate or reinitiate a self-sustaining cancer immunity-cycle, enabling the cycle to amplify and propagate. Because checkpoints and inhibitors are built into each step that negatively regulate cancer immunity-cycle, every step of the cancer immunity-cycle can be targeted to augment the effects of the host immune system against cancer. In the past decade, various types of cancer immunotherapies that act at different stages of this cycle to augment immune responses have been developed such as cancer vaccinations and monoclonal antibodies. Among numerous approaches of cancer immunotherapies, immune checkpoint blockade (ICB) has shown remarkable success. The strategy of ICB therapy is to inhibit negative

immune regulatory checkpoints such as programmed cell death-1 (PD-1) and cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) to unleash pre-existing anti-tumor T cell immune responses, causing durable remissions in some patients with previously untreatable tumors, such as advanced refractory malignant melanoma[9–11]. In 2013, cancer immunotherapy was declared as the "breakthrough of the Year", and cancer immunotherapy swept Nobel for medicine in 2018[12,13]. Since cancer immunotherapy is becoming recognized as a valid and critically important approach to treat patients with cancer, more and more research and clinical investigation efforts have become dedicated to advancing safe and effective immunotherapy approaches[14].

Adoptive cell transfer immunotherapies

Adoptive cell transfer (ACT), also known as cellular immunotherapy, is a technique in which cells are collected from a cancer patient, expanded and activated *ex vivo*, and then transferred back into the patient[15]. Many types of cells have been used for ACT both in human and veterinary medicine.

Lymphokine-activated killer (LAK) cell therapy was one of the first cell transfers. In the 1980s, Rosenberg *et al.* reported that LAK cells could be generated from human peripheral blood leukocytes using interleukin (IL)-2 and that they showed anti-tumor effects both *in vitro* and *in vivo*[16–19]. LAK cell therapy has also been investigated in veterinary medicine[15,20]. Several papers reported LAK cells were generated from autologous peripheral blood lymphocyte (PBL) activated by anti-cluster of differentiation (CD) 3 antibody and IL-2 and the resulting cell product showed antitumor effects against several types of canine cancer cell lines *in vitro*, such as thyroid carcinoma, malignant melanoma and hepatocarcinoma[21–24]. It has also been reported that sequential administration of LAK cells promoted host immune cell proliferation and interferon (IFN)-γ levels in the serum without severe adverse effects[21]. However, the clinical efficacy of LAK therapy is limited and has not been established as a monotherapy both in human and veterinary medicine. One possible reason for the failure of clinical

application is that LAK cells do not have the ability to recognize antigens expressed by cancer cells and unable to kill them. Thus, it was thought as important to use T cells that can recognize antigens expressed by tumor cells for adoptive cell transfer therapy.

Thereafter, tumor infiltrating T lymphocyte (TIL) became the focus of attention for another type of cells for cell transfer on behalf of LAK cells. Because TILs were originated from T cells primed and activated via cancer antigen presentation by APCs, they can potentially kill cancer cells through the recognition of MHC-presented antigen via T cell receptor (TCR). In fact, increased $CD8^+$ TILs within tumor tissue have been known as a good prognostic marker for various types of cancer in human including melanoma[25,26], breast cancer[27,28] and colorectal tumors[29]. In 1988, Rosenberg *et al.* firstly demonstrated that the infusion of autologous TIL isolated and expanded from melanoma tissue, induced the regression of metastatic melanoma in patients treated concurrently with high doses of IL-2[30–32]. Although TIL therapy has demonstrated objective tumor response in dramatic response in around 50% of human patients with metastatic melanoma, adaptation of TIL therapy is currently limited for other types of tumor due to low availability of TILs and the complexity of their production[33,34]. On the other hand, TILs have yet to be well characterized in dogs, though several reports have implied the association of T lymphocyte infiltration with progression of canine cancers[35–37]. Moreover,

TILs for ACT therapy have not been established in dogs with any types of cancer. Thus, it has yet to be succeeded for TIL therapy to be widely applied clinically in both human and veterinary medicine.

The barriers to clinical application of TIL therapy include T cell exhaustion caused by both extrinsic negative regulatory pathways (such as immunoregulatory cytokines) and cell intrinsic negative regulatory pathways (such as PD-1)[38], leading to failure of adequate T cell expansion *ex vivo*. Also, even if TILs were originated from T cells primed and activated via cancer antigen presentation by APCs, TILs may not recognize cancer cells properly due to low affinity of TCR for self-antigen or downregulation of MHC on cancer cells. Furthermore, it is limitation of TIL therapy that tumor removal by surgery is required to obtain TILs from tumor tissues. Thus, the use of TILs for ACT has many limitations for wide application in human and canine patients with cancer. Therefore, other approaches which overcome the limitation of TILs are required for success of ACT immunotherapies.

Chimeric antigen receptor (CAR)-T cell therapy

To overcome the challenges of LAK and TIL therapies, such as no or low specificity for tumor cells and exhaustion of T cell by tumor microenvironment, gene-engineered T cells has been extensively developed to create activated T cells that have cancer specificity. Chimeric antigen receptors (CARs) are synthetic T cell receptors composed of an extracellular single-chain variable fragment (scFv) derived from the variable heavy and variable light domains of a given antibody, linked to transmembrane and intracellular signaling domains composed of the intracellular component of CD3z plus co-stimulatory molecules such as CD28 or 4-1BB. (Fig. B)[39]. T cells expressing the CAR on their surface, get activated following binding of the CAR to a specific antigen on the surface of cancer cells, leading to proliferation, expansion and effector function against the target cells[40]. The advantages of CAR-T cells to other cellular approaches are (1) MHC-independence, (2) in cis costimulatory and activation activity and (3) expanded targets for tumor recognition[39].

CAR-T cell therapy has recently shown great success in human medicine, especially for hematological malignancies. CD19 targeting CAR-T (CD19-CAR-T) cells have produced remarkable durable clinical responses in human patients with relapsed/refractory B cell malignancies, and CD19-CAR-T cells were approved for

children with refractory and relapsed acute lymphoblastic leukemia (ALL) and adults with large B cell lymphoma (BCL) in 2017[41]. Since the remarkable success with CD19-CAR-T therapy were reported, there has been much research into CAR-T cell approaches to overcome some of the barriers to feasible and successful therapy and expand the types of tumor that can be treated using CAR-T cell therapy to effectively treat more cancer patients.

Considering the remarkable success of CAR-T therapy in human medicine, CAR-T therapy may also be a promising treatment for cancer patients in veterinary medicine. CAR-T cell therapy has been explored in dogs for hematological and solid tumors *in vitro* and *in vivo* respectively[42–45]. However, this approach is still in infancy in this species and more basic research is required to optimize this approach for use in canines with cancer.

In order to achieve clinical application of CAR-T cell therapy in canine patients with cancer, there are a number of barriers that must be overcome. First of all, stable and high CAR expression on canine T cells is required for the administration of CAR-T cell to canine patients. Secondly, persistence of CAR-T cells in patients are required for durable remissions for cancer patients. Thirdly, ideal targets for CAR-T cells on many different cancer types need to be identified. These barriers need to be overcome in both

hematologic and solid tumors, but a number of other strategies are also required in solid tumors, such as ensuring that CAR-T cells injected into the patient are able to infiltrate the tumor tissues and resist immunosuppression in tumor microenvironment. All of these barriers need to be overcome in order to realize the clinical application of CAR-T cell therapy in dogs.

Purpose of this study

The purpose of this study was to optimize CAR-T cell therapy for canine malignant tumors. In Chapter 1, CAR-T cells were generated from T cells from healthy dogs using a retroviral vector to obtain stable CAR expression on the surface of canine T cells, and antigen-specific effector function of the CAR-T cells against target cells was evaluated. In Chapter 2, the effect of different co-stimulatory domains on CAR-T cell function was compared to choose the most suitable CAR construct for clinical trials for canine patients with B cell lymphoma. Since fundamental information to generate canine CAR-T cells was revealed in Chapters 1 and 2, target antigens for CAR-T cells were explored to expand cancer types for further application of CAR-T cell therapy in dogs in Chapter 3.

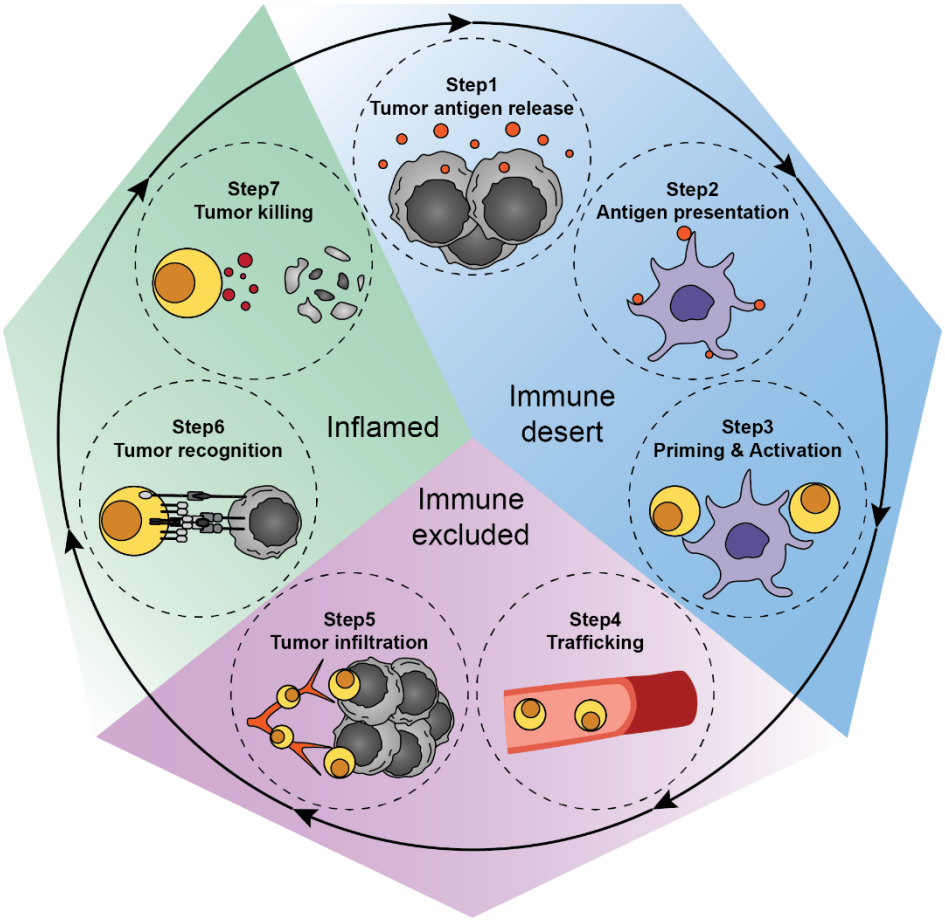

Fig. A Cancer immunity-cycle

Chen *et al.* proposed the cancer immunity-cycle composed of 7 serial steps initiated by recognizing cancer cell antigens created through oncogenesis (step 1)[8]. Antigen presenting cells (APC) (e.g. dendritic cells (DC)) take up antigens and present them on major histocompatibility class (MHC) molecules to T cells (step 2), leading to priming and activation of effector T cell response (step 3). Then, the activated effector T cells traffic to the tumor site (step 4) and infiltrate the tumor tissue (step 5). Finally, T cells recognize their cognate peptides bound to MHC class I via their T cell receptor (TCR) (step 6) and kill cancer cells (step 7).

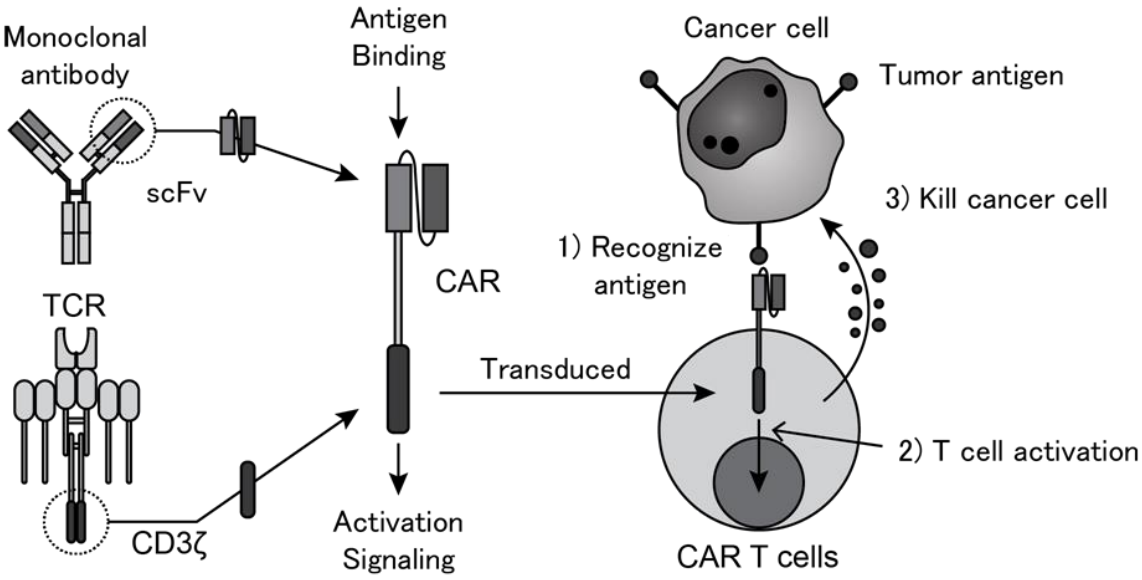

Fig. B Chimeric antigen receptor-T cell therapy

Chimeric antigen receptors (CARs) are synthetic T cell receptors composed of an extracellular single-chain variable fragment (scFv) derived from the variable heavy and variable light domains of a given antibodies, linked to transmembrane and intracellular signaling domains which induce naïve T cell activation[39]. CAR-T cells, the T cells expressing CAR on their surface, get activated by binding to a specific antigen on the surface of cancer cells through the CAR, leading to proliferation, expansion and effector function against the target cells[40].

Chapter 1

Development of functional canine chimeric antigen receptor (CAR)-T cells using retroviral vectors

Introduction

CAR-T cells are generated from patient-derived T cells using gene transfer technology[46]. Generally, patients undergo apheresis to isolate their mononuclear cells, T cells are isolated and activated, and then T cells are transduced with the CAR by gene transfer technology. Then, CAR-T cells are expanded *ex vivo* in the presence of certain cytokines such as IL-7 and IL-15 and introduced back into the patients, where they seek out target tumor cells and kill them. In the process of CAR-T cell manufacturing, T cell transduction with the CAR is one of the important steps to ensure that there are sufficient numbers of CAR-T cells to administrate to the patient.

Gene transfer technology is indispensable for generating CAR-T cells and the technology has rapidly advanced in the last two decades[47]. Currently, T cells are transduced with CAR using either viral or non-viral methods in the human clinic[48]. In dogs, Panjwani *et al.* developed an expansion methodology that yields large numbers of canine T cells from dogs and succeeded in generating canine CAR-T cells using mRNA electroporation[42]. In this study, the CD20 targeting CAR was highly expressed on the surface of both canine $CD4^+$ and $CD8^+$ T cells 24 hours post-electroporation, but a gradual reduction in CAR frequency was observed over 14 days, indicating transient surface expression of CAR after electroporation[42]. Although CAR expression were

transient, the electroporated CD20-targeted CAR-T cells exhibited antigen-specific IFN-γ secretion and cytotoxicity against cell lines expressing CD20[42]. Moreover, injection of the CAR-T cells was well tolerated in a first-in-canine study, it led to a modest, but transient, antitumor activity in one dog with B cell lymphoma[42]. These results provided proof of concept, but the results suggested that stable CAR expression will be necessary for durable clinical remission for dogs with B cell lymphoma.

Viral vectors are most commonly used for gene transfer in human CAR-T products. Lentiviral vectors from the family *Retroviridae* are now the most commonly used vectors for gene transfer worldwide[49]. Because viral gene transfer has the capacity to stably integrate genetic material into the host genome, the technique could be applied to generate canine CAR-T cells to get stable CAR expression on the surface of canine T cells. In Chapter 1, the use of a retroviral vector was explored to transduce canine T cells with a CD20-targeting CAR to generate canine CAR-T cells. And the antigen-specific effector function of these CAR-T cells was evaluated *in vitro*.

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Chapter 2: Comparison of the effects of different co-stimulatory domains on canine CAR-T cell function

Introduction

T cells requires three signals to get completely activated against cancer within the immune synapse formed between APCs and T cells[69]. Signal 1 is the basis for T cell specificity for antigen, comprising the recognition of antigen/major histocompatibility complexes by the T-cell receptor (TCR). Signal 2 has role to ramping up and amplifying the T cell response given through signal 1, caused by co-stimulatory signal which occurs when co-stimulatory molecules on the surface of the T cell bind to their ligands to antigen-presenting cells[70]. In the absence of the second co-stimulatory signal, primary TCR stimulation fails to activate T cells and induces anergy[71]. So far, a variety of co-stimulatory molecule have been found and most co-stimulatory receptors belong to the immunoglobulin (Ig) (e.g. CD28, ICOS) or tumor necrosis factor receptor (TNFR) (e.g. 4-1BB, OX40, CD27) superfamilies. Signal 3 is the signal which caused by the secretion of cytokines by APCs, which can help amplify and expand the T cell response against cancer. These three signals are crucial to induce a good T cell response against cancer.

In the beginning of CAR-T development, the structure of CAR was composed of an antibody-derived scFv linked to a hinge region and transmembrane domain followed by CD3z signaling domain. The CARs, now termed 'first-generation' CARs, get activated by recognition of target antigen and exhibit cytotoxicity against target cells, but the

CAR-T cells failed to proliferate well or to lead long-term antitumor responses[72]. Thereafter, the CARs which contained one of the co-stimulatory domains between a transmembrane domain and CD3z signaling domain were developed. In humans and mice, T cells containing the CARs, now termed 'second-generation' CARs, have demonstrated greater expansion of CAR-T cells leading to long-term antitumor responses[73]. Many co-stimulatory molecules have incorporated to second-generation CARs such as CD28, 4-1BB, ICOS, OX40 or CD27, and increasing number of researches have investigated fundamental characteristics of co-stimulatory signaling to improve function of CAR-T cells[74,75]. For instance, CD28 is the one of the co-stimulatory molecules which commonly used for CAR-T cells and belongs to Ig superfamily. In both mice and human studies, T cells incorporating the CD28 domain underwent a greater degree of expansion and persisted for longer periods, leading improved antitumor efficacy, compared to first-generation CAR which has no co-stimulatory domain[76,77]. Another example of co-stimulatory domains is 4-1BB, which belongs to TNFR superfamily. CARs incorporating a 4-1BB co-stimulatory domain favors development of central memory T cells, which appears to favor longer CAR-T cell persistence, in contrast to those incorporating a CD28 domain elicits an effector memory T cell phenotype. 4-1BB-based CAR-T cell therapy targeting CD19 has shown great success in human medicine, and

the CAR-T cell therapy received FDA approval for children with refractory and relapsed ALL and adults with large BCL[78,79]. Furthermore, an ICOS co-stimulatory domain, which belong Ig superfamily as CD28 is also thought to have advantages for CAR-T cell therapy. Gueden *et al.* reported ICOS-based CARs augmented the effector function and *in vivo* persistence of Th17 polarized cells, compared to CAR incorporating CD28 or 4-1BB. They also reported $CD4^+$ T cells expressing an ICOS-based CAR significantly increased the persistence of co-injected $CD8^+$ T cells. Based on these findings, different co-stimulatory signaling within a CAR would have different effect on the function, persistence and cell subset development of CAR-T cells. Therefore, it would be important to choose optimal co-stimulatory molecule for CAR-T cells, but it still remains unknown which co-stimulatory molecule is the best to achieve durable remissions in human patients with cancer.

Although selecting optimal co-stimulatory domain for canine CAR-T cells is important, information surrounding the effects of different co-stimulatory domains on canine T cell function is lacking. Thus, I hypothesized that as in humans and mice, different co-stimulatory domains have different effect on the functions of canine CAR-T cells, such as proliferation and cytokine productions. To address this hypothesis, I designed a first-generation canine CAR (no co-stimulatory domain) and second-

generation CARs that contain one of three co-stimulatory domains; CD28, 4-1BB or ICOS, and evaluated their effect on the function of CAR-T cells against canine cancer *in vitro*.

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Chapter 3: Exploring tumor antigen to widen the range of tumor types for canine CAR-T cell therapy

Introduction

A major advantage of the CAR strategy is that antigen recognition occurs via the CAR's scFv without the requirement of peptide presentation of by MHC. In short, CAR-T cells can exert effector functions following the CAR interacting directly with surface antigen on tumor cells[86]. Since the recognition and subsequent clearance of tumor cells are dependent on the CAR, the selection of target antigen is important. Ideally, target antigens should be exclusively expressed on the surface of cancer cells to prevent on target, off-tumor side effects of therapy. On exception to this is if the target antigen is shared normal cell types that are nonessential, such as CD19 which is expressed on healthy B cells as well as malignant B cells.

Although CAR-T cell therapy has shown great success in human medicine for hematological malignancies such as CD19 targeting CAR-T cells for patients with ALL and BCL, the efficacy of CAR-T cells therapy in solid tumors has been disappointing[40,81]. One of the challenges for CAR-T cell therapies particularly in solid tumors is the difficulty in identification of an ideal target antigen[40]. Solid tumors are heterogeneous in composition and antigens expressed on tumor cells are often expressed on normal cells as well, which makes it difficult to find an ideal target antigen.

Recently, increasing numbers of target antigens are being reported for some

solid tumors in human medicine and many clinical trials of CAR-T cell are ongoing for human patients with solid tumors[86]. There are mainly three main types of target antigens; tumor specific antigen, tumor associated antigen or cancer germline antigens, based on their expression patterns[86]. Firstly, tumor specific antigen is the antigen which expressed only on malignant tissue such as epidermal growth factor receptor variant III (EGFRvIII). Although it is unlikely to cause on target, off-tumor side effects, it is also difficult to identify tumor specific targets due to limited pool of possible proteins. Secondly, tumor associated antigen is the antigen which expressed at low levels on normal cells while expressed at a higher level on tumor cells such as human epidermal growth factor receptor 2 (HER2). Although it increases the likelihood that target antigens can be identified, extensive preclinical validation is required to determine safety due to possibility to cause on target, off-tumor side effects. And finally, cancer germline antigen is the antigen which frequently expressed on cancer cells, expressed on embryonic cells and minimally expressed on normal cells in healthy tissues such as IL-13Rα2. Cancer germline antigen expression is largely restricted to the testis or ovary, limiting on target, off-tumor side effects due to restricted expression in adult somatic tissue. However, it is difficult to identify cancer germline antigen due to limited pool of possible proteins, and application of CARs targeting the antigens could result in the

destruction of healthy reproductive tissue even though it may not be life-threatening damage. More recently, it has been thought that the target antigens include not only proteins listed above, but also proteins that exhibit particular protein post-translational modifications such as carbohydrate chains (e.g. MUC1).

As opposed to human medicine, while CD20 targeting CAR-T cells are being evaluated in the canine oncology clinic, there are no ongoing clinical studies using CAR-T cell therapies for canine patients with solid tumors. To realize the potential of CAR-T cells for the treatment of solid tumors as well as hematological malignancies in dogs, target antigens for CAR-T cells therapy need to be identified in canine solid tumors. Since the generation of canine CAR-T cells was optimized (described in Chapter 1 and Chapter 2), the next step necessary to widen the range of tumor types that could be treated using CAR T cell therapy is to explore possible target antigens in various solid tumors in dogs. Considering the reports in human and the availability of cross-reactive antibodies for use in dogs, two antigens were chosen as candidates for further investigation; human epidermal growth factor receptor 2 (HER2) and podoplanin (PDPN). Studies designed to evaluate these two target antigens for CAR-T cell therapy of solid tumors are described in section 1 and 2 respectively.

Section 1: HER2

Introduction

HER2 is a transmembrane receptor tyrosine kinase and one of the members of the human epidermal growth factor receptor (EGFR) family[87]. HER2 plays a crucial role in the development of normal tissue such as in the nervous system and mammary glands; however, it has also been known to regulate oncogenesis[87,88]. HER2 amplification and protein overexpression have been reported in various human cancers, including breast, gastric, ovarian, lung and thyroid cancers[89–93]. Because HER2 overexpression is related to carcinogenesis and aggressive phenotype in some types of cancers, HER2 is used as both a diagnostic marker and therapeutic target[94]. HER2 expression has also been reported in several canine tumors such as mammary gland tumor (MGT), osteosarcoma (OSA), and transitional cell carcinoma (TCC)[95–97].

In human medicine, several HER2-targeted therapies have been developed including antibodies and low molecular substances. Trastuzumab (Herceptin), a humanized antibody directed against the extracellular domain of human HER2, is used to prevent the metastasis of human breast cancer[98]. Lapatinib, a small molecule acting as a dual epidermal growth factor receptor (EGFR) and HER2 tyrosine kinase inhibitor, is approved for the treatment of human patients with HER2-positive breast cancers[99,100].

It has also been reported in veterinary medicine that HER2-targeted therapies like lapatinib and a recombinant human HER2 expressing *Listeria* vaccine showed an anti-tumor effect on canine HER2-positive cancers[101,102].

HER2 has been suggested as a promising target antigen for CAR-T cell therapy because HER2 expressed at low levels on normal cells while expressed at a higher level on tumor cells. In fact, HER2 targeting CAR-T cells have been developed and are currently being evaluated in clinical trials in human patients with HER2-postive tumors. In 2014, HER2-CAR-T cells were developed for dogs and using *in vitro* studies, the authors demonstrated cytotoxicity against canine OSA expressing HER2. Moreover, since HER2 expression has been reported in canine MGT and TCC, clinical application of HER2-targeted CAR-T cells is expected for these tumors as well. However, HER2 expression in other tumor types remains unknown. Therefore, HER2 expression in other tumor types are required to be evaluated to widen the range of tumor types that could be treated using HER2-targeted CAR T cell therapy. The objective of the study in section 1 was to evaluate HER2 expression in anal sac gland carcinoma (ASAC), primary lung cancer (PLC) and thyroid cancer (TC) in dogs. These cancer types were chosen by a pre-screening experiment where HER2 expression were evaluated in various types of canine cancers by IHC using several samples each.

Materials and Methods

Sample Collection

To evaluate mRNA expression, fresh tumor tissue (ASAC, PLC and TC) and normal tissues (normal anal sac, lung and thyroid) were obtained from dogs with tumors and healthy beagles, respectively. The tumor tissue was surgically removed from canine patients at the Veterinary Medical Center of the University of Tokyo between April 2017 and December 2018. To investigate the immunohistochemical expression, paraffin-embedded tumor tissue of canine patients was analyzed. The tissue was surgically removed between January 2011 and December 2016. The tissue samples were diagnosed by veterinary pathologists certified by the Japanese College of Veterinary Pathologists at the Department of Veterinary Pathology at the University of Tokyo.

Permission for the collection and usage of resected tissue for this study was obtained from owners. Healthy dog tissue was obtained from healthy beagles euthanized for other experiments. All procedures were approved by the Animal Care and Use Committee at the University of Tokyo (Approval number: P17-074).

Semi-quantification of mRNA of HER2

Total RNA was extracted and reverse transcribed using a transcriptase

(ReverTra Ace, Toyobo, Osaka, Japan). Real-time polymerase chain reaction (PCR) was performed using a premix reagent (THUNDERBIRD SYBR qPCR Mix, Toyobo), specific primers for HER2 (Forward primer: 5'-CAGCCCTGGTCACCTACAA-3', Reverse primer: 5'-CCACATCCGTAGACAGGTAG-3'), and a real-time PCR system (StepOnePlus, Thermo Fisher Scientific, Waltham, MA, USA). mRNA expression was analyzed using standard curve method, and expression values were normalized using GAPDH (Forward primer: 5'-TGACACCCACTCTTCCACCTTC-3', Reverse primer: 5'—CGGTTGCTGTAGCCAAATTCA-3') as an internal control.

Immunohistochemistry (IHC)

Canine tumor tissue was retrospectively evaluated. All samples were fixed in 10% neutral-buffered formalin, embedded in paraffin wax, and cut into 4 μm-thick serial sections. Paraffin-embedded tumor sections were dewaxed and rehydrated in xylene and graded ethanol. Antigen retrieval was then performed using Dako Target Retrieval Solution, pH 9.0 (Agilent Technologies, Santa Clara, CA, USA) by microwaving for 15 min at 750 W. After washing with tris-buffered saline containing 0.1% Tween 20 (TBS-t), endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 10 min. Specimens were then washed with TBS-T and incubated in 8% skimmed milk for 1

hr at 20-28°C to reduce non-specific binding before overnight incubation with primary antibodies, which included mouse IgG1 anti-HER2 monoclonal antibodies (clone: CB-11, Leica biosystems, Wetzlar, Germany) diluted 1:40, at 4°C in a humidified chamber. A negative control was incubated with purified mouse IgG1, κ isotype antibody (Clone: MG1-45, BioLegend, San Diego, CA, USA), under identical conditions. After washing with TBS-T, sections were incubated with a horseradish peroxidase (HRP)-conjugated anti-mouse antibody (Envision+ System-HRP Labelled Polymer; K4001; Agilent Technologies) for 30 min at 20-28°C. Thereafter, the sections were washed with TBS-T, incubated with 3,3' diaminobenzidine (Dojindo Laboratories, Rockville, MD, USA) solution for 3 min, and counterstained with Mayer's hematoxylin. For the negative control, sections were subjected to the same procedures. The results were quantified according to the recent guidelines proposed by the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP)[103,104].

Cell line

Six canine TCC cell lines (Love, Sora, TCCUB, OMTCC, MCTCC and NMTCC), six canine mammary grand tumor (MGT) cell lines (CTBp, CTBm, CHMp, CHMm, CIPp and CIPm), three canine OSA cell lines (POS, HMPOS and HOS), two canine MM cell

lines and MDCK were used to evaluate HER2 expression on the surface of cell lines. TCC cell lines were maintained in RPMI-1640 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 5 mg/L gentamicin (Sigma-Aldrich, MO, USA) at 37°C in a humidified atmosphere with 5% CO2. MGT, OSA, MM and MDCK cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin (Wako, Osaka, Japan) at 37°C in a humidified atmosphere with 5% CO2.

Flow cytometry

Fresh tumor and metastatic lymph node tissues were minced with scalpels and digested in 1 mg/ml collagenase I (Wako, Osaka, Japan) with 0.5% bovine serum albumin at 37°C for 2 hr. Undigested tissue was removed by filtration using a 100 µm mesh. Cell lines were trypsinized into single cell suspensions, and then used for the following procedure. Then, cells were stained using a humanized anti-human HER2 specific monoclonal antibody trastuzumab, which was previously shown to have cross-reactivity to HER2 on the surface of canine cancers[105]. Cells were incubated with 200 µl of 10 µg/ml trastuzumab (Herceptin; Roche, Hertfordshire, UK) or without trastuzumab as a

negative control for 30 min at 4°C, followed by three washes in fluorescence-activated cell sorting (FACS) buffer (phosphate-buffered saline containing 5% fetal bovine serum). Then, cells were incubated with anti-human IgG APC antibodies (BioLegend) diluted 1:25 for 30 min at 4°C, and washed with FACS buffer. Negative controls were also subjected to the same procedures. After live cells were gated by forward scatter/side scatter (FSC/SSC), fluorescence was measured and analyzed using a flow cytometer and its related software (BD FACSVerse™, Becton-Dickinson, Franklin Lakes, NJ, USA). Staining positivity was confirmed by gates sets on the negative control.

Statistical analysis

All data are shown as means ± standard deviation (SD). The statistical methods and software used were Welch's t-test performed using the R software (R Development Core Team, 2018) and the beeswarm R package (Version 0.2.3, 2016). A $p < 0.05$ was considered statistically significant.

Results

HER2 expression in ASAC

To compare mRNA expression levels of HER2 in ASAC and normal anal sac tissues, real-time PCR were performed to evaluate mRNA expression in these tissues. HER2 mRNA expression in ASAC tissue was found to be significantly higher than that in normal anal sac tissue (HER2/GAPDH: 0.67 vs 0.05; range: 0.26–1.68, 0.02–0.24; $p < 0.05$). Although various expression levels of HER2 were confirmed in ASAC tissue, it was elevated in all ASAC tissues compared to normal anal sac tissue (Fig. 3-1-1).

To evaluate protein expression of HER2 in patients with ASAC, paraffin-embedded ASAC tissue samples were retrospectively evaluated by IHC. A total of 25 ASAC tissue samples were collected from dogs (2 intact males, 13 castrated males, 5 intact females, and 5 spayed females) of different breeds (10 Miniature Dachshunds, 4 Chihuahuas, 3 Toy Poodles, 2 Golden Retrievers, 1 Beagle, 1 French Bulldog, 1 Jack Russell Terrier, 1 Siberian Husky, 1 Shiba Inu and 1 Shetland Sheepdog). The mean age was 11.4 ± 2.1 (population SD), range: 6.8–15.2 years. In all, 15 tumors were located on the left side and 10 on the right side; 5 cases (20%) had stage 1 disease, and 9 (36%), 8 (32%), 2 (8%), and 1 (4%) had stage 2, 3a, 3b, and 4, respectively. Immunohistochemical staining revealed broad expression of HER2 in ASAC tissue. HER2-positive ASAC tissue

demonstrated strong and broad staining of the circumferential membrane of ASAC cells and were negatively stained on the peri-tumor normal cells (Fig. 3-1-1). HER2 expression in ASAC tissues was scored based on the ASCO/CAP guidelines (Table 3-1-1) to evaluate the staining pattern of HER2. Representative images of the staining pattern for each score are shown in Fig. 3-1-1. Analysis of HER2 expression score demonstrated that 6 samples (24%) scored 3+, 14 (56%) scored 2+, and 5 (20%) scored 1+, with no samples scoring 0 (Table 3-1-2, 3-1-5). In total, 80% of canine ASAC tissue was positive for HER2 (scored > 2+). Statistical analysis revealed that there was no relationship between HER2 score and clinical data such as location, TNM classification and clinical stage.

To investigate whether trastuzumab recognizes canine HER2 on the surface of ASAC cells, flow cytometric analysis was performed on fresh primary ASAC and metastatic lymph node tissues. In the result, most cells in the primary and metastatic tumor tissues were stained by trastuzumab (Fig. 3-1-1).

HER2 expression in PLC

To compare mRNA expression levels of HER2 in PLC and normal lung tissues, real-time PCR was performed. In the result, PLC tissues showed higher levels of HER2 expression than normal tissues although there was significant variation in expression of

HER2 mRNA in tumor tissues compared with normal lung tissues (Fig. 3-1-2). Of 6 PLC samples, 5 expressed higher HER2 mRNA expression than any normal samples.

To evaluate protein expression of HER2 in patients with PLC, paraffin-embedded PLC tissue samples were retrospectively evaluated by IHC. Sixteen PLC tissue samples were collected from 16 dogs (5 castrated male, 3 intact male, 7 spayed female, and 1 intact female) of different breeds (5 Chihuahuas, 4 Miniature Dachshunds, 2 Shih Tzu, 1 Beagle, 1 English Pointer, 1 Maltese, 1 Saluki, and 1 Toy Poodle). The mean age was 12.3 ± 1.3 years (population standard deviation), ranging from 10.2 to 15.1 years. Three tumors were located in the right cranial lung lobe, 3 in the right middle lung lobe, 2 in the right caudal lung lobe, 1 in the left cranial lung lobe, and 7 in the left caudal lung lobe (Table 3-1-3). PLC tissues demonstrated strong and broad HER2-positive staining of the circumferential membrane of PLC cells and negative or weakly positive staining on peri-tumor normal cells and normal lung tissues via IHC staining (Fig. 3-1-2). HER2 expression in PLC tissues was scored based on the ASCO/CAP guidelines (Table 3-1-1) to evaluate the staining pattern of HER2. Representative staining patterns of each score are shown in Fig. 3-1-2. HER2 expression score analysis demonstrated that 3 samples (19%) scored 3+; 8 samples (50%), 2+; 5 samples (31%), 1+; and 0 sample (0%), 0 (Table 3-1-3, 3-1-5). In total, 69% of PLC tissues were HER2 positive (scored > 2+).

Statistical analysis revealed that there was no relationship between HER2 expression scores and clinical characteristics such as location and TNM classification (Table 3-1-3).

HER2 expression in TC

To evaluate protein expression of HER2 in patients with TC, paraffin-embedded TC tissue samples were retrospectively evaluated by IHC. Twenty-one TC tissue samples were collected from 21 dogs (8 castrated males, 8 intact males, 5 spayed females) of different breeds (4 Beagles, 4 Miniature Dachshunds, 3 mixed breeds, 2 Labrador Retrievers, 2 Welsh Corgi Pembrokes, 2 Yorkshire Terriers, 1 Chihuahua, 1 Pekinese, 1 Schipperke, and 1 Shih Tzu). The mean age was 10.3 ± 2.3 years (population standard deviation), ranging from 6.5-13.8 years. Thirteen (62%) tumors were unilateral; 6 (29%), bilateral; and 2 (10%), ectopic. One case had stage 1 disease, and 13 (62%), 5 (24%), and 2 (10%) cases had stage 2, 3, and 4, respectively (Table 3-1-4). HER2-positive TC tissues demonstrated strong broad staining of the circumferential membrane of TC cells and negative staining on peri-tumor normal cells via immunohistochemical staining (Fig. 3-1-3). HER2 expression in TC tissues was scored based on the ASCO/CAP guidelines (Table 3-1-1) to evaluate the staining pattern of HER2. Representative staining patterns of each score are shown in Fig. 3-1-3. HER2 expression score analysis demonstrated that

4 samples (19%) scored 3+; 6 samples (29%) scored 2+; 7 samples (33%) scored 1+; and 4 samples (19%) were negative (Table 3-1-4, 3-1-5). Almost half (48%) of the TC tissues were HER2 positive (scored > 2+). Statistical analysis revealed that there was no relationship between HER2 score and clinical data such as location, TNM classification and clinical stage (Table 3-1-4).

Surface expression of HER2 in canine various cancer cell lines

To evaluate surface expression of HER2 on the various canine cell lines, cells were analyzed by flow cytometric analysis using a humanized anti-human HER2 specific monoclonal antibody trastuzumab, which was previously shown to have cross-reactivity to HER2 on the surface of canine cancers[105]. The cell lines which were analyzed by flow cytometry were canine TCC, MGT, OSA and MM cell lines, and all of these cancer types were previously reported to express HER2 by IHC or WB. Consistent with these reports, surface expression of HER2 was detected by trastuzumab in all types of cancer cell lines including TCC, MGT, OSA and MM (Fig. 3-1-4).

Discussion

In human medicine, HER2 is a well-established therapeutic target and is known to be overexpressed in a variety of human tumors. In fact, HER2-targeted antibody therapies such as trastuzumab and pertuzumab have been widely applied clinically in human cancers expressing HER2[98,106]. From the success of HER2-targeted therapies to date, HER2 is also thought to be a potential target for CAR-T cells as well. In fact, there are many ongoing clinical trials of HER2-targeted CAR-T cell worldwide for their clinical application[107].

The expression of HER2 is also known to occur in several cancers such as TCC, OSA and MGT in dogs[95–97], and some approaches targeting HER2 showed anti-tumor effect against canine cancers expressing HER2 such as lapatinib, neratinib and trastuzumab and recombinant *Listeria* vaccines[101,108,109]. Given that many HER2-targeted therapies showed anti-tumor effect against canine cancers in laboratory studies, HER2 could be a promising target for CAR-T cell therapy in canine tumors as well. In fact, CAR-T cell therapy targeting HER2 were reported to show cytotoxicity against canine OSA expressing HER2[110]. Although other types of canine cancer could be targeted by HER2-targeted CAR-T cells, it still remains unclear what canine cancer types are potentially targeted HER2-targeted CAR-T cells.

Therefore, in Section 1, HER2 expression was investigated in several different cancer types, which were chosen according to preliminary experiment, to determine whether HER2-targeted CAR-T cells therapy could be effective against these tumor types in the dog. HER2 expression was evaluated in three types of canine cancers including ASAC, PLC and TC. As for ASAC, HER2 mRNA expression of ASAC tissues was significantly higher than that of normal anal sac tissues. IHC analysis confirmed HER2 protein expression in ASAC tissues, with approximately 80% of canine patients being positive, while HER2 expression was also detected in normal anal sac tissues, the apocrine glands and squamous epithelia. The surface expression of HER2 was detected by trastuzumab, which has been reported to be cross-reactive previously, and the expression was confirmed in cancer cells obtained from both primary and metastatic tissues. As for PLC, 5 of 6 PLC samples expressed higher HER2 mRNA expression than any normal samples, but the difference was not significant. However, IHC analysis confirmed 69% of canine PLC samples were positive for HER2 protein, while HER2 was weakly detected in normal lung tissue. As for TC, although only IHC analysis was performed due to lack of fresh tumor samples, HER2 protein expression was confirmed in 48% of canine patients, while weak HER2 expression was found in normal thyroid tissues. These results suggest that these cancer types expressing HER2 would be

candidates for HER2-targeted CAR-T cell therapy in canine patients.

While the clinical application of HER2-targetd CAR-T cells therapy is promising, damage to normal tissues by HER2-targeted CAR-T cells, which is known as on-target, off-tumor toxicity, is major concern. In fact, first clinical use of HER2-targeted CAR-T cells in human with a HER2 positive colon cancer resulting in a serious adverse event following injection of HER2-targeted CAR-T cells[111]. The patient developed respiratory distress within 15 minutes of receiving CAR-T cells, then multiple cardiac arrests occurred, eventually leading to death[111]. The reason why this severe event happened is thought to be because HER2-targeted CAR-T cells recognized normal tissues expressing HER2, especially lung epithelium[111]. Since HER2 expression was also confirmed in normal canine tissues including the lung, anal sac and thyroid tissues, on target, off-tumor cytotoxicity by HER2-targeted CAR-T cells may also occur in canine patients. To overcome on-target, off-tumor toxicity, next-generation CAR-T cells, with improved tumor specificity through targeting of multiple antigens, were recently developed in human medicine, and this strategy would reduce on-target, off-tumor toxicity by HER2-targeted CAR-T cells in canine patients.

As a conclusion to section 1, because HER2 expression was confirmed in canine ASAC (80%), PLC (69%) and TC (48%), HER2-targeted CAR-T cells might be effective

for these cancer as well as the cancer types which previously reported to express HER2 such as canine OSA, MGT and TCC. However, HER2 expression was also confirmed in several normal tissues, so it would be major concern for on-target, off-tumor toxicities of HER2-targeted CAR-T cells. Further studies are required to evaluate whether HER2-targeted CAR-T cells would show anti-tumor effect against HER2-expressed ASAC, PLC and TC *in vitro* and *in vivo*, and to overcome on-target, off-tumor toxicities which would potentially be caused by HER2-targeted CAR-T cells.

Table 3-1-1. Human epidermal growth factor receptor 2 scoring methods proposed by the American Society of Clinical Oncology/College of American Pathologists

	Description
3+	Circumferential membrane staining that is complete, intense, and in >10% of tumor cells
2+	Circumferential membrane staining that is incomplete and/or weak/moderate and in >10% of tumor cells or Complete and circumferential membrane staining that is intense and in <10% of tumor cells
1+	Incomplete membrane staining that is faint/barely perceptible and in >10% of tumor cells
0	No staining is observed or Membrane staining that is incomplete and is faint/barely perceptible and in <10% of tumor cells.

Table 3-1-2. Characteristics of the dogs with anal sac gland carcinoma and HER2 expression

No	Breed[a)]	Age (years)	Sex[b)]	Location[c)]	TNM classification			Clinical stage	IHC[d)] score (primary tumors)	IHC score[e)] (lymph nodes)
					T	N	M			
1	MD	9.3	MC	R	T1	N0	M0	1	2+	–
2	JRT	13.7	MC	R	T2	N0	M0	1	2+	–
3	MD	9.8	MC	L	T2	N0	M0	1	2+	–
4	MD	12.2	MC	R	T2	N0	M0	1	2+	–
5	MD	12.4	M	L	T2	N0	M0	2	1+	–
6	MD	13.3	MC	L	T2	N0	M0	2	1+	–
7	GR	11.5	MC	L	T2	N0	M0	2	1+	–
8	TP	11.7	F	L	T2	N0	M0	2	2+	–
9	FB	9.4	MC	L	T2	N0	M0	2	2+	–
10	MD	9	MC	L	T2	N0	M0	2	2+	–
11	MD	10.4	M	R	T2	N0	M0	2	2+	–
12	GR	9.9	MC	R	T3	N0	M0	2	2+	–
13	CH	12	MC	L	T2	N0	M0	2	3+	–
14	MD	12.4	FS	L	T2	N1b	M0	3a	1+	N/A
15	TP	9.6	F	R	T1	N2b	M0	3a	2+	3
16	TP	6.8	F	L	T2	N1b	M0	3a	2+	N/A
17	SS	11.3	FS	L	T2	N1b	M0	3a	2+	N/A
18	MD	11.1	MC	L	T1	N1b	M0	3a	3+	N/A
19	CH	14.6	MC	L	T1	N1b	M0	3a	3+	N/A
20	BG	14.3	F	L	T2	N1b	M0	3a	3+	N/A
21	CH	15.2	MC	L	T2	N1b	M0	3a	3+	N/A
22	SI	11.3	FS	R	T2	N1b	M0	3b	2+	2
23	SH	11.8	F	R	T2	N1b	M0	3b	3+	3
24	MD	13.4	FS	R	T1	N2b	M1	4	1+	N/A
25	CH	8.3	FS	R	T1	N0	M1	4	2+	–

a) MD: Miniature Dachshund, JRT: Jack Russell Terrier, GR: Golden Retriever, TP: Toy Poodle, FB: French Bulldog, CH: Chihuahua, SS: Shetland Sheepdog, BG: Beagle, SI: Shiba Inu, SH: Siberian Husky

b) MC: Male castrated, M: Male, FS: Female spayed, F: Female

c) R: Right, L: Left

d) IHC: Immunohistochemistry

e) –: No lymph node metastasis, N/A: Not available due to non-resectable tumor

Table 3-1-3. Characteristics of the dogs with canine primary lung cancer and HER2 expression

No.	Breed[a)]	Age (years)	Sex[b)]	Location	TNM classification			Classification	IHC[c)] score
					T	N	M		
1	CH	13.2	MC	Right cranial	T1	N0	M0	Adenocarcinoma	1+
2	CH	11.5	FS	Right middle	T1	N0	M0	Adenocarcinoma	1+
3	TP	11.3	MC	Left caudal	T1	N1	M0	Adenocarcinoma	1+
4	SAL	11.8	M	Left caudal	T1	N1	M0	Adenocarcinoma	1+
5	SHI	10.2	FS	Left caudal	T1	N0	M0	Adenocarcinoma	2+
6	CH	12.3	MC	Left caudal	T1	N0	M0	Adenocarcinoma	2+
7	MLT	13.6	MC	Left caudal	T1	N0	M0	Adenocarcinoma	2+
8	SHI	11.8	MC	Left cranial	T1	N0	M0	Adenocarcinoma	2+
9	MD	11.2	FS	Right middle	T1	N0	M0	Adenocarcinoma	2+
10	CH	11.2	M	Right cranial	T1	N0	M0	Adenocarcinoma	2+
11	CH	14.2	F	Right cranial	T1	N0	M0	Adenocarcinoma	2+
12	MD	15.1	M	Right middle	T1	N0	M0	Adenocarcinoma	3+
13	MD	12.3	FS	Left caudal	T2	N0	M1	Adenocarcinoma	3+
14	BG	13	FS	Right caudal	T1	N0	M0	Alveolar carcinoma	3+
15	EP	12.8	FS	Right caudal	T1	N0	M0	Bronchial cell carcinoma	2+
16	MD	12.3	FS	Left caudal	T1	N0	M0	Squamous cell carcinoma	1+

a) CH: Chihuahua, TP: Toy Poodle, SAL: Saluki, SHI: Shih Tzu, MLT: Maltese, MD: Miniature Dachshund, BG: Beagle, EP: English Pointer

b) M: male, MC: male castrated, F: female, FS: female spayed

c) IHC: immunohistochemistry

Table 3-1-4. Characteristics of the dogs with thyroid cancer and HER2 expression

No	Breed[a)]	Age (years)	Sex[b)]	Location	TNM classification			Clinical stage	IHC score
					T	N	M		
1	MD	12.1	M	Ectopic	T1	N0	M0	1	2+
2	CH	11.6	FS	Right	T2	N0	M0	2	0
3	SCHI	13.1	MC	Left	T2	N0	M0	2	0
4	SHI	6.8	M	Left	T2	N0	M0	2	1+
5	BG	9.2	MC	Bilateral	T2	N0	M0	2	1+
6	BG	7.9	M	Bilateral	T2	N0	M0	2	1+
7	YT	12	MC	Ectopic	T2	N0	M0	2	2+
8	WCP	12.7	MC	Bilateral	T2	N0	M0	2	2+
9	LR	11.3	MC	Right	T2	N0	M0	2	2+
10	LR	7.3	FS	Right	T2	N0	M0	2	3+
11	MD	11.9	MC	Right	T2	N0	M0	2	3+
12	MIX	11.5	FS	Right	T2	N0	M0	2	3+
13	MD	8.3	FS	Right	T2	N0	M0	2	3+
14	PE	7.1	M	Bilateral	T2	N2	M0	2	2+
15	MIX	11	M	Right	T3	N0	M0	3	0
16	MD	6.5	FS	Right	T3	N0	M0	3	1+
17	WCP	10.7	MC	Left	T3	N0	M0	3	1+
18	YT	11.9	M	Bilateral	T3	N1	M0	3	0
19	BG	10.4	M	Right	T3	N1	M0	3	1+
20	MIX	13.8	MC	Right	T2	N0	M1	4	1+
21	BG	8.5	M	Bilateral	T3	N0	M1	4	2+

a) MD: Miniature Dachshund, CH: Chihuahua, SCHI: Schipperke, SHI: Shih Tzu, BG: Beagle, YT: Yorkshire Terrier, WCP: Welsh Corgi Pembroke, LR: Labrador Retriever, PE: Pekingese

b) M: Male, MC: Male castrated, F: Female, FS: Female spayed

Table 3-1-5. HER2 score in canine ASAC, PLC and TC tissues

	Negative		Positive	
Score	0	1+	2+	3+
ASAC (n=25)	0 (0%)	5 (20%)	14 (56%)	6 (24%)
PLC (n=16)	0 (0%)	5 (31%)	8 (50%)	3 (19%)
TC (n=21)	4 (19%)	7 (33%)	6 (29%)	4 (19%)

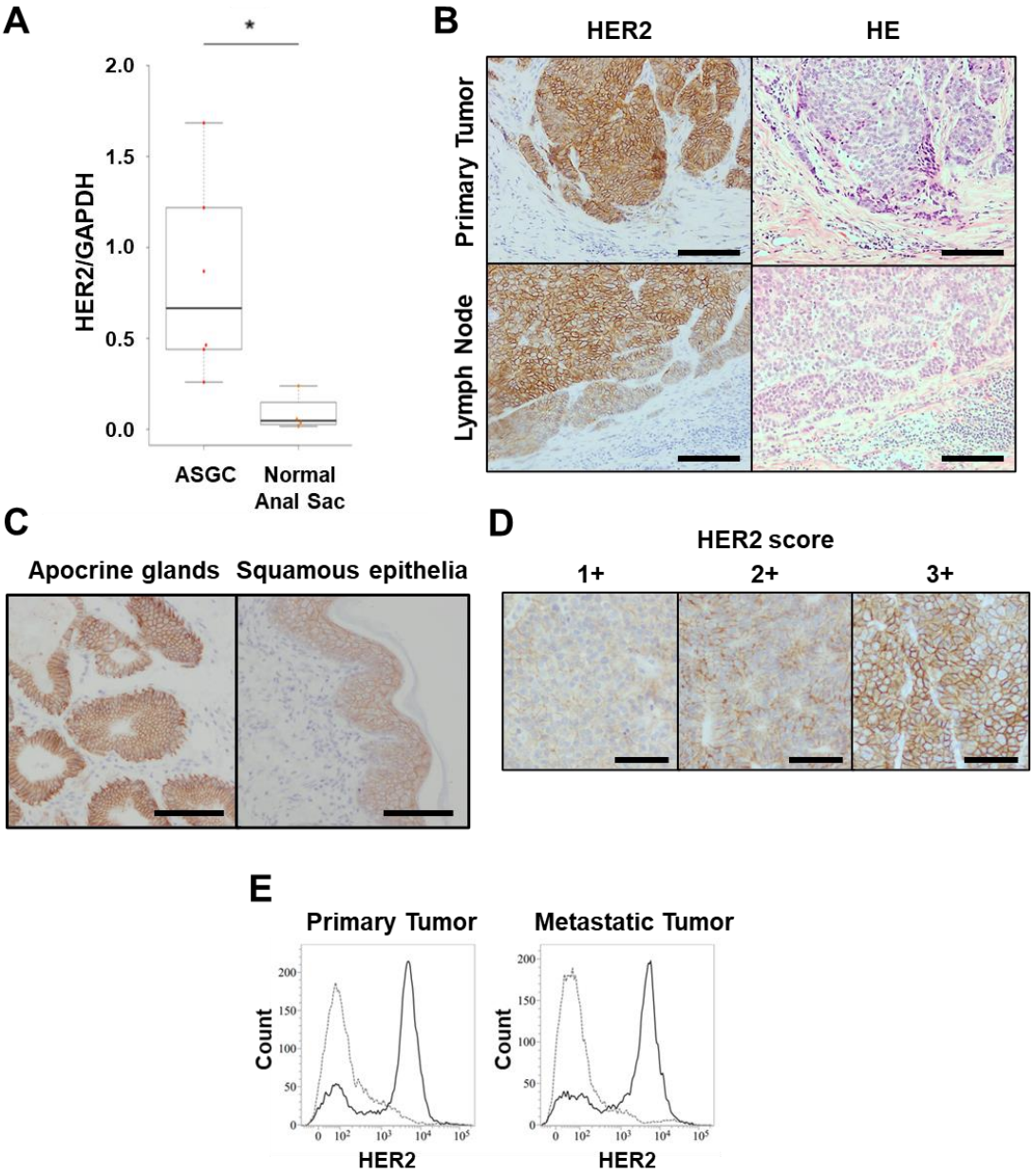

Fig. 3-1-1 HER2 expression in anal sac adenocarcinoma

(A) HER2 mRNA expression in anal sac adenocarcinoma (ASAC) and normal anal sac tissues. HER2 mRNA expression in ASAC tissue was significantly higher than that in the normal anal sac tissues ($p < 0.05$). (B) Representative images of the staining pattern of HER2 in canine ASAC. Circumferential membrane staining that is complete and intense in canine ASAC cell in the primary tumor and metastatic lymph node. Scale bar, 100μm. (C) Representative images of the staining pattern of HER2 in canine normal anal sac tissues, the apocrine glands and squamous epithelia. Scale bar, 100 μm. (D) HER2

scoring of canine ASAC based on ASCO/CAP. Representative images of the staining pattern of HER2 in canine ASAC. Scale bar, 50μm. (E) Surface expression of HER2 on the tumor cells in primary tumor and metastatic lymph node by flow cytometric analysis (gray dotted line: negative control, black solid line: trastuzumab). Cells were gated on live cells based of forward scatter (FSC)/side scatter (SSC).

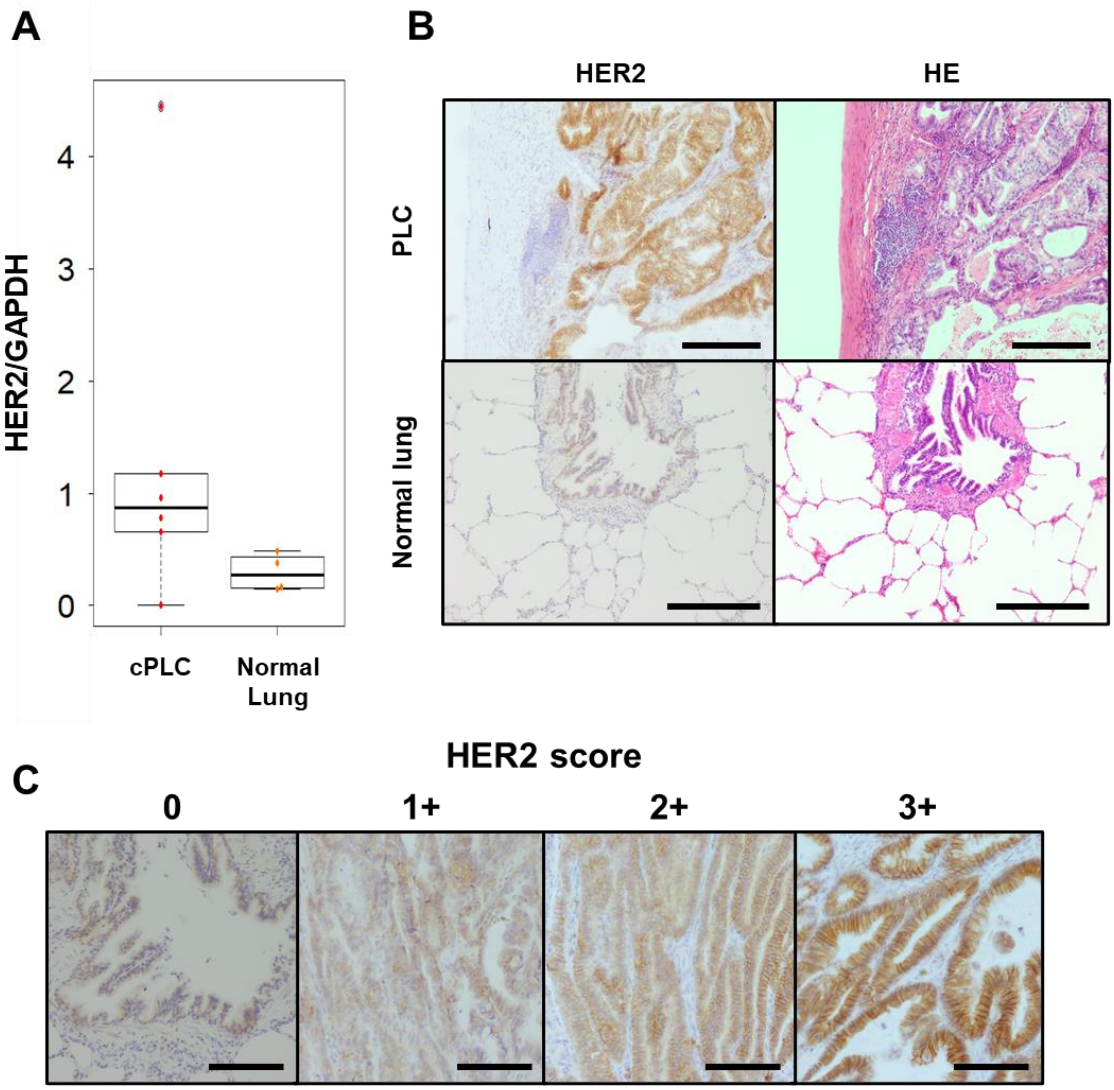

Fig. 3-1-2 HER2 expression of canine primary lung cancer

(A) HER2 mRNA expression in primary lung cancer (PLC) and normal lung tissues. Of 6 canine PLC samples, 5 expressed higher HER2 mRNA than any normal samples. (B) Representative images of the staining pattern of HER2 in canine PLC and normal lung tissue. Circumferential membrane staining is complete and intense in canine PLC. Scale bar, 200μm. (C) HER2 scoring of canine PLC based on ASCO/CAP. Representative images of the staining pattern of HER2 in canine PLC. Scale bar, 50μm.

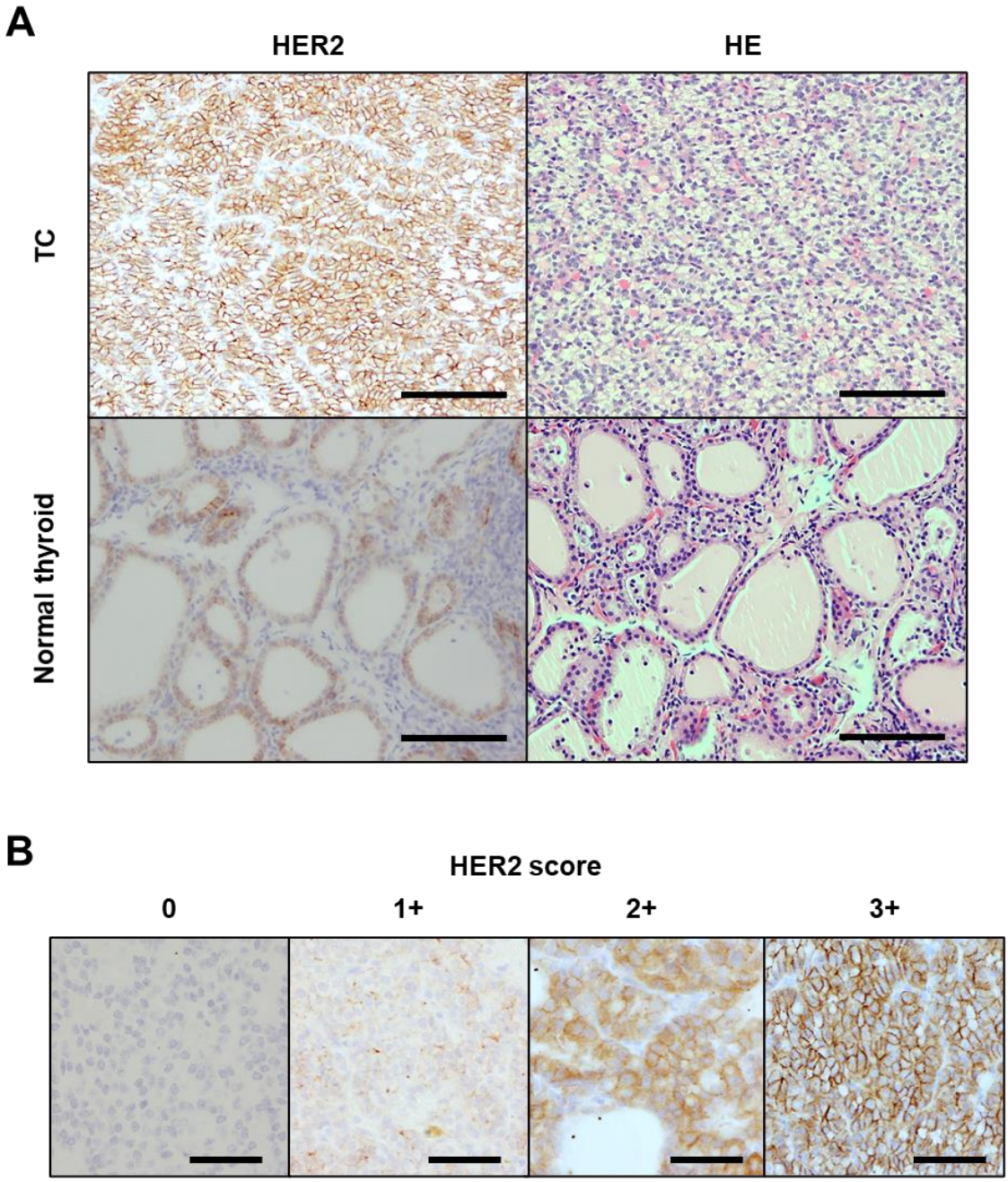

Fig. 3-1-3 HER2 expression of thyroid cancer

(A) Representative images of the staining pattern of HER2 in canine thyroid cancer (TC) and normal thyroid tissue. Circumferential membrane staining is complete and intense in canine TC. Scale bar, 200μm. (B) HER2 scoring of canine TC based on ASCO/CAP. Representative images of the staining pattern of HER2 in canine PLC. Scale bar, 50μm.

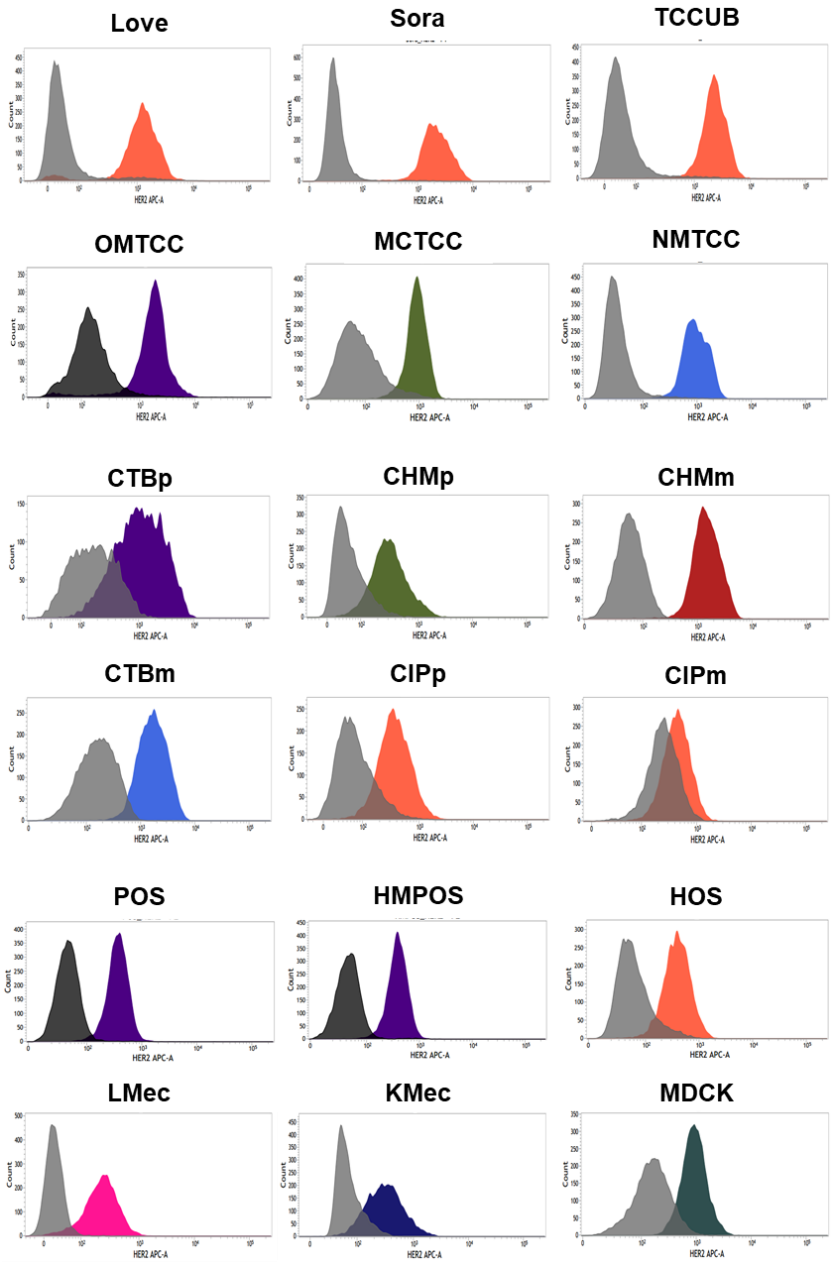

Fig. 3-1-4 Surface expression of HER2 on various canine tumor cell lines

After trypsinization of canine cancer cell lines, cells were incubated with trastuzumab or without trastuzumab as negative control, followed by wash in FACS buffer. Then, cells were incubated with anti-human IgG APC antibodies. Surface expression of HER2 were analyzed after live cells were gated by forward scatter/side scatter (FSC/SSC). Trastuzumab detected canine HER2 on the surface of canine cancer cell lines including TCC, MGT, OSA and MM. Staining positivity was confirmed by negative control (grey line).

Section 2: PDPN

Introduction

Podoplanin (PDPN) is a type I transmembrane sialoglycoprotein which has essential functions in the development of various tissues including alveoli, heart and lymphatic vascular system[112]. PDPN is also known to induce platelet aggregation through interacting with C-type lectin-like receptor 2[113]. In human medicine, the expression of PDPN was widely reported in various cancer types such as squamous cell carcinoma (SCC), osteosarcoma (OSA) and germinoma[114–116]. PDPN expression was not only reported in cancer cells but also cancer-associated fibroblasts (CAFs) that constitute major component of the cancer stroma and associated with poor prognosis of human patients with some types of cancer[117–120]. Recently, PDPN was reported to promote tumor progression and distant metastasis by facilitating tumor cell migration and inducing platelet aggregation[121–123]. Moreover, PDPN expression in cancer tissues were associated with poor prognosis of patients with some types of cancer[121–123]. Although PDPN is expressed in various types of cancer and the expression promote cancer malignancy, PDPN is not thought as a target antigen for CAR-T cells. Since PDPN is expressed in lymphatic endothelial cells, alveolar type I cells and renal podocytes[112,113], there is a major concern about on target, off-tumor side effects caused by the damage to these cells in normal tissues[112,114,124].

Aberrant glycosylation of glycoproteins is now recognized as one of the features of neoplastic transformation[125]. Taking advantage of the feature, Kato *et al.* recently developed a cancer-specific monoclonal antibody (CasMab) which binds aberrant glycosylated PDPN in tumor tissues, but does not bind to normal PDPN[126]. Therefore, even if the glycoproteins could not be a target antigen due to concerns about damage to normal cells, it can be potential target for CAR-T cells by targeting only aberrant glycosylated glycoproteins. Kato *et al.* have developed several anti-dog PDPN monoclonal antibodies as well, and reported that one of the antibodies, PMab-38 recognized PDPN in cancer tissues such as canine SCC and MM but not recognized PDPN in normal tissues such as lymphatic endothelial cells alveolar type I cells[127–129]. The results suggested PMab-38 specifically recognizes an aberrantly glycosylated PDPN in these two canine cancers. Thus, PDPN might be a promising target by using PMab-38 for canine cancers such as SCC and MM and that scFv developed from PMab-38 might be able to re-direct canine T cell specificity using CAR technology to develop PDPN-targeted CAR-T cells.

However, it is unclear whether PDPN in cancer tissue was specifically recognized by PMab-38 in various types of cancers other than canine SCC and MM. It has also been shown that there is no positive staining for PMab-38 in any systemic normal tissues. Thus, the objective of this section was to evaluate the staining with

PMab-38 by immunohistochemical analysis in various types of canine cancers and systemic normal tissues for investigating whether scFv of PMab-38 would be applied to CAR-T cell therapy for canine patients with tumors.

Materials and Methods

Sample Collection

Paraffin-embedded tumor tissues were collected at the Veterinary Medical Center of the University of the Tokyo between 2012 and 2016. These tumor tissues were pathologically evaluated at the Department of Veterinary Pathology at the University of Tokyo. Systemic normal tissues were obtained from healthy dogs that euthanized for other experiments. All procedures were approved by the Animal Care and Use Committee at the University of Tokyo (Approval number: P17-074).

Cell lines and cell culture

Twelve canine MM cell lines (CMM1, CMM2, CMM7, CMM8, CMM9, CMM10, CMM11, CMM12, KMeC, LMeC, CMeC1 and CMeC2)[130–134], Chinese hamster ovary (CHO)-K1 and canine PDPN-expressing CHO-K1 with an N-terminal PA tag (CHO/dPDPN) were generated for negative and positive controls respectively. CMM7, CMM8, CHO-K1 and CHO-dPDPN[135,136] were maintained in RPMI-1640 (FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% heat-inactivated FBS and 5 mg/L gentamicin (Sigma-Aldrich) at 37°C in a humidified atmosphere with 5% CO2. All canine MM cell lines but CMM7 and CMM8 were maintained in DMEM supplemented

with 10% FBS, 100 units/ml penicillin, and 0.1 mg/ml streptomycin (Wako) at 37°C in a humidified atmosphere with 5% CO2.

Immunohistochemistry

Canine tumor tissue was retrospectively evaluated. All samples were fixed in 10% neutral-buffered formalin, embedded in paraffin wax, and cut into 4 μm-thick serial sections. Paraffin-embedded tumor sections were dewaxed and rehydrated in xylene and graded ethanol. Antigen retrieval was then performed using Dako Target Retrieval Solution, pH 9.0 (Agilent Technologies, Santa Clara, CA, USA) for 30 min in boiling water. After washing with tris-buffered saline containing 0.1% Tween 20 (TBS-t), endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 10 min. Specimens were then washed with TBS-T and incubated in 8% skimmed milk for 1 hr at 20-28°C to reduce non-specific binding before overnight incubation with primary antibodies at 4°C in a humidified chamber. Primary antibodies included the mouse IgG1 anti-PDPN (PMab-38[128]) antibody, an anti-pan-PDPN (PMab-48[136]) antibody, or the anti-α-smooth muscle actin (α-SMA) antibody (clone: 1A4, Agilent Technologies). A negative control was incubated with purified mouse IgG1, κ isotype antibody (Clone: MG1-45, BioLegend, San Diego, CA, USA), under identical conditions. After washing with TBS-T, sections were

incubated with a horseradish peroxidase (HRP)-conjugated anti-mouse antibody (Envision+ System-HRP Labelled Polymer; K4001; Agilent Technologies) for 30 min at 20-28°C. Thereafter, the sections were washed with TBS-T, incubated with 3,3' diaminobenzidine (Dojindo Laboratories, Rockville, MD, USA) solution for 3 min, and counterstained with Mayer's hematoxylin. For the negative control, sections were subjected to the same procedures. When any stained images of cells in tumor tissue were confirmed, the specimens were determined to be positive.

Flow cytometry

Cells were harvested by brief exposure to 0.25% trypsin/1mM EDTA (Nacalai Tesque, Inc.). After three washes in FACS buffer, cells were incubated with 2 μg/ml PMab-38 or without purified mouse IgG1, κ isotype antibody (Clone: MG1-45, BioLegend) as negative control for 30 min at 4°C, followed by three washes in FACS buffer. Then, cells were incubated with FITC conjugated anti-mouse IgG1 antibodies diluted 1:80 for 30 min at 4°C, and washed with FACS buffer. Negative controls were also subjected to the same procedures. After live cells were gated by FSC/SSC, fluorescence was measured and analyzed using a flow cytometer and its related software (BD FACSVerse™). Staining positivity was confirmed by negative control.

Results

PMab-38 specifically recognized PDPN in SCC but not PDPN in normal lung tissues

To evaluate whether PMab-38 specifically recognized PDPN in SCC, the staining patterns of IHC were compared between PMab-38 or anti-pan-PDPN antibody. Type I alveolar cells in normal lung are reported to express PDPN, and this expression was confirmed by staining with anti-pan-PDPN antibody (PMab-48). However, type I alveolar cells were not stained with PMab-38 (Fig. 3-2-1). On the other hand, tumor cells in SCC tissues positively stained with both PMab-38 and anti-pan-PDPN (PMab-48) antibody, and the staining pattern was equivalent between PMab-38 and anti-pan-PDPN antibody (Fig. 3-2-1). These results suggested PMab-38 specifically recognized PDPN in SCC and the structure of epitope in PDPN may be altered by cancer-specific aberrant glycosylation.

Positive staining of PMab-38 was detected in canine tumor cells and CAFs in various types of tumors

To investigate what tumor types expressed PDPN that specifically recognized by PMab-38, IHC analysis using PMab-38 was performed in 123 specimens of various tumor types. In the results, some parts of tumor cells were stained with PMab-38 in 82%

(9/11) of SCC, 82% (9/11) of PAC, 40% (4/10) of FSA, 15% (2/13) of MAC, 10% (1/10) of OSA and 10% (1/10) of ASAC samples (Fig. 3-2-2, Table. 3-2-1). Large area (> 50%) of tumor cells were stained in some specimens of SCC and PAC samples, while small area (<10%) of tumor cells were stained in the other types of tumor tissues. Normal tissues surrounded tumor area were not stained by PMab-38 in all specimens.

To evaluate whether PDPN was recognized by PMab-38 in tumor stroma, the same specimens were analyzed by IHC. In the results, some parts of stroma cells were stained with PMab-38 in 92% (11/12) of TCC, 90% (9/10) of ASAC, 82% (9/11) of SCC, 55% (6/11) of PAC, 25% (2/8) of PNST, 22% (2/9) of TC, 22% (2/9) of THY, 20% (3/13) of MAC and 10% (1/10) of HCC samples (Table. 3-2-1). In FSA and OSA samples, tumor cells and stromal cells were not evaluated separately due to difficulty of distinguishing these cells. Since these stromal cells which stained with PMab-38 were suspected to be fibroblast, especially (CAFs), the expressions of α-SMA, one of the markers for CAFs, were evaluated in these tumor tissues[137,138]. Some parts of stromal cells in tumor cells were stained with α-SMA, and some parts of α-SMA positive cells also showed positive staining for PMab-38. However, the stained area for α-SMA and PMab-38 were not completely consistent.

PMab-38 detected surface expression of PDPN on canine MM cell lines

To determine whether PMab-38 detected PDPN on the surface of canine tumor cells, flow cytometric analysis was performed for canine MM cell lines, which were previously reported to express PDPN by IHC[139]. As previously reported, PMab-38 recognized PDPN on the surface of CHO-dPDPN, but not CHO-K1. Also, PMab38 detected PDPN on the surface on some of canine MM cell lines. In the results, 4 of 12 canine cell lines were positive for PMab-38 by flow cytometric analysis (Fig. 3-2-3).

PDPN was not detected by PMab38 in most normal tissues.

To evaluate whether PMab-38 recognized PDPN in systemic normal tissues, IHC was performed using PMab-38 in systemic normal tissues. In the results, normal cells in most systemic tissues were not stained with PMab-38 including lymph node and lung tissues which previously reported to express PDPN (Fig. 3-2-4). However, podocytes in kidney tissues were weakly stained with PMab-38, although staining intensity of podocytes were lower than that of SCCs.

Discussion

The expression of PDPN has been reported in many types of human tumors[114–116]. Moreover, it has been reported that PDPN is involved in tumor progression in some types of tumor[117–120], suggesting that it may be a potential therapeutic target. However, PDPN is also known to be expressed in normal cells such as lymphatic endothelial cells and alveolar type I cells[112], raising concerns about side effects due to damage to these cells. Therefore, PDPN was thought to be unsuitable as a therapeutic target for CAR-T cell therapy, which possibly cause strong damages against cells expressing the target antigen. Against this background, it was reported that aberrant glycosylation of glycoproteins happened in tumor tissues, raising possibility to avoid injury to normal cells by specifically targeting glycoproteins with aberrant glycosylated glycoproteins in tumor tissues[125]. Recently, Kato *et al.* developed CasMab technology and succeeded in developing an antibody that specifically recognizes aberrant glycosylated PDPN in tumor tissues, thereby potentially allowing for the practical use of CAR-T cell therapies specifically targeting aberrant glycosylated PDPN on tumor cells[126]. Furthermore, the same group have also developed several antibodies against canine PDPN, and one of the antibodies, PMab-38, showed high affinity to PDPN in tumor tissues[139,140].

The purpose of the study in section 2 was to investigate whether PMab-38 specifically bound PDPN in canine tumor tissues, and to identify what tumor types would be targeted by CAR-T cells using scFv of PMab-38. Firstly, IHC analysis was performed for SCC and normal lung tissues using PMab-38 and anti-pan-PDPN antibody. In the results, anti-pan-PDPN antibody detected PDPN of both type I alveolar cells and SCC cells, while PMab-38 recognized PDPN of canine SCC but not PDPN of type I alveolar cells, suggesting that PMab-38 may specifically bind to aberrant glycosylated PDPN in tumor tissues. Then, IHC analysis using PMab-38 was performed in various types of canine tumors. In the results, PDPN of tumor cells were recognized by PMab-38 in about 80% of SCC and PAC samples and a portion of the FSA, MAC, OSA and ASAC samples. Since both SCC and PAC are common cancers in dogs and are not able to be treated by surgery alone in some cases, PDPN-targeted CAR-T cell therapy using PMab-38 may be one of the promising therapeutic options to treat these cancers. Moreover, flow cytometric analysis revealed 4 of 12 canine MM cell lines were recognized by PMab-38, suggesting that the scFv of PMab-38 would be used for CAR-T cell targeting PDPN.

Interestingly, PDPN of stromal cells were also recognized by PMab-38 in about 90% of ASAC and TCC samples and a portion of PNST, TC, THY and HCC samples, similar to the reports in humans[115–117]. Some of stromal cells which recognized by PMab-

38 were positive for α-SMA, suggesting these stromal cells were CAFs, which are known to play an important role in tumor by secreting cytokines and growth factors[138,141]. Since CAFs are known to promote tumor progression, not only cancer cells but also CAFs can be therapeutic targets. In fact, many types of CAFs-targeted therapies have recently been developed, and some of the therapies have shown anti-tumor effects *in vitro* and *in vivo*. Moreover, PDPN-positive CAFs were associated with increased lymphatic invasion and lymph node metastasis in some types of cancer including melanoma[117,119], suggesting that it may be important to target CAFs expressing PDPN. Thus, the tumor types of which PDPN of CAFs were recognized by PMab-38 such as ASAC and TCC can be targeted by CAR-T cells using scFv of PMab-38.

In order to investigate whether any normal cells in systemic tissues were not recognized by PMab-38, IHC were performed using PMab-38 for systemic normal tissues. In the results, PMab-38 did not recognize PDPN in lymph node and lung tissues, where PDPN was known to be expressed. Although weak positive staining of PMab-38 was not confirmed in almost all tissues, PDPN of podocyte in renal glomerulus was recognized by PMab-38. One of the reasons why PDPN of podocyte was recognized by PMab-38 may be because glycosylation of the epitope that recognized by PMab-38 in PDPN is similar between podocytes and cancer cells unlike lymphatic endothelial cells and alveolar type

I cells. Since PMab-38 did recognize PDPN in lymphatic endothelial cells and type I alveolar cells[112], PDPN-targeted CAR-T cells of which scFv are derived from PMab-38 would have less possibility of on-target, off-tumor toxicity than the CAR-T cells of which scFv are derived from anti-pan-P DPN antibody. However, there are concerns about the side effects of targeting PDPN in podocytes targeted by the CAR-T cells of which scFv are derived from PMab-38.

In conclusion, PDPN would be a one of the promising targets for CAR-T cells by selectively targeting aberrant glycosylated PDPN, and scFv of PMab-38 can be used to develop CAR-T cells targeting aberrant glycosylated PDPN. Further studies are required to evaluate anti-tumor effects of PDPN-targeted CAR-T cell which are developed using the VH and VL chains of PMab-38 reformatted as a scFv to confirm effector function and no on-target, off-tumor toxicity for safety.

Table 3-2-1. Immunohistochemical analysis of PMab-38 staining in various types of canine tumor

Tumor types[a)]	Samples	Tumor cells (%)	Stroma cells (%)[b)]
SCC	11	9 (82)	9 (82)
PAC	11	9 (82)	6 (55)
FSA	10	4 (40)	ND
MAC	13	2 (15)	3 (20)
OSA	10	1 (10)	ND
ASAC	10	1 (10)	9 (90)
TCC	12	0 (0)	11 (92)
PNST	8	0 (0)	2 (25)
TC	9	0 (0)	2 (22)
THY	9	0 (0)	2 (22)
HCC	10	0 (0)	1 (10)
HSA	11	0 (0)	0 (0)

a) SCC: Squamous cell carcinoma, PAC: Pulmonary adenocarcinoma, MAC: Mammary adenocarcinoma, FSA: Fibrosarcoma, OSA: Osteosarcoma, ASAC: Anal sac adenocarcinoma, TCC: Transitional cell carcinoma, PNST: Peripheral nerve sheath tumor, TC: Thyroid carcinoma, THY: Thymoma, HCC: Hepatocellular cell carcinoma, HSA: Hemangiosarcoma.

b) ND: Not-determined

Table 3-2-2. Recognition of PDPN by PMab-38 on canine MM cell lines analyzed by flowcytometric analysis

Cell line	PDPN expression
CMM1	+
CMM2	−
CMM7	−
CMM8	+
CMM9	−
CMM10	−
CMM11	+
CMM12	+
KMec	−
LMec	−
CMec1	−
CMec2	−

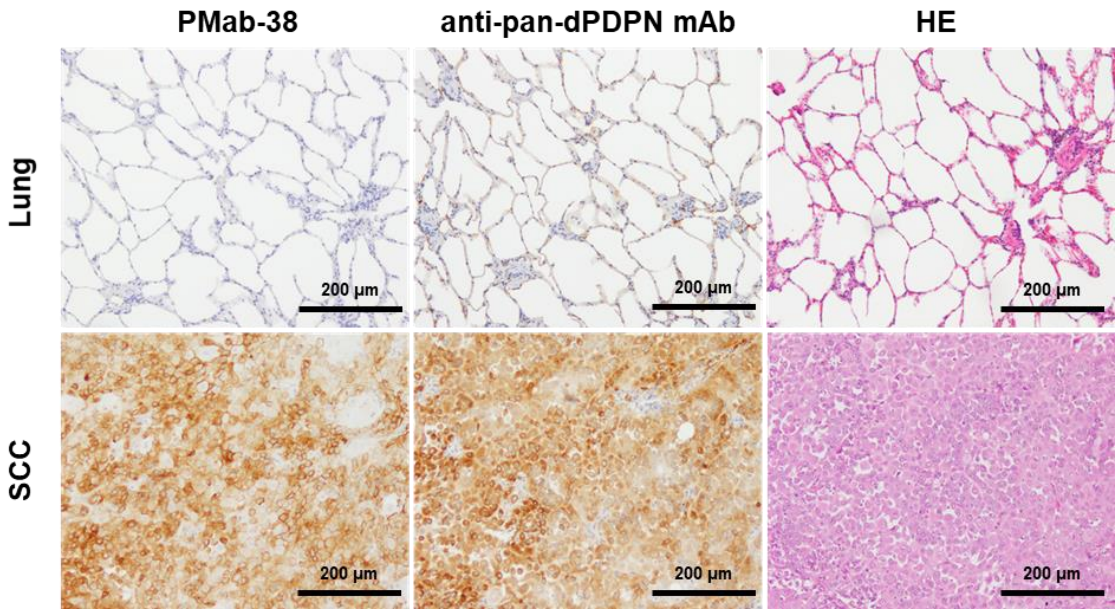

Fig. 3-2-1 Characteristics of staining pattern by PMab-38 and anti-pan-PDPN antibody (PMab-48)

Representative staining patterns of IHC using PMab-38 and anti-pan-PDPN monoclonal antibody (PMab-48) in normal lung and SCC tissues are shown.

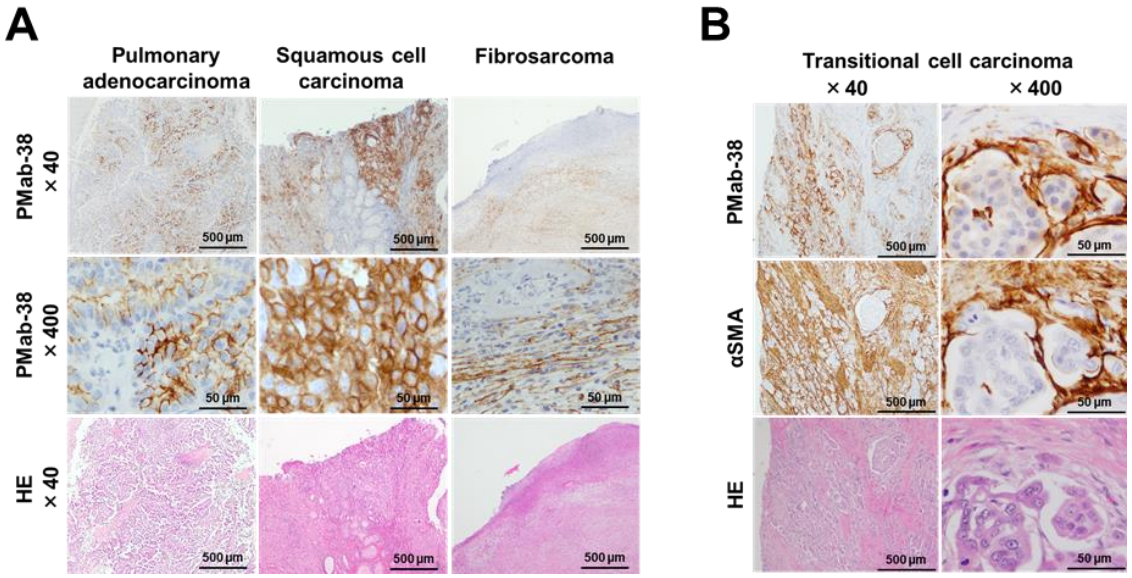

Fig. 3-2-2 Evaluation of the staining pattern of PMab-38 in tumor cells and stromal cells

(A) Representative staining patterns of IHC analysis using PMab-38 in PAC, SCC, and FSA. (B) Representative staining pattern of IHC analysis using PMab-38 and anti-α-SMA antibodies in TCC.

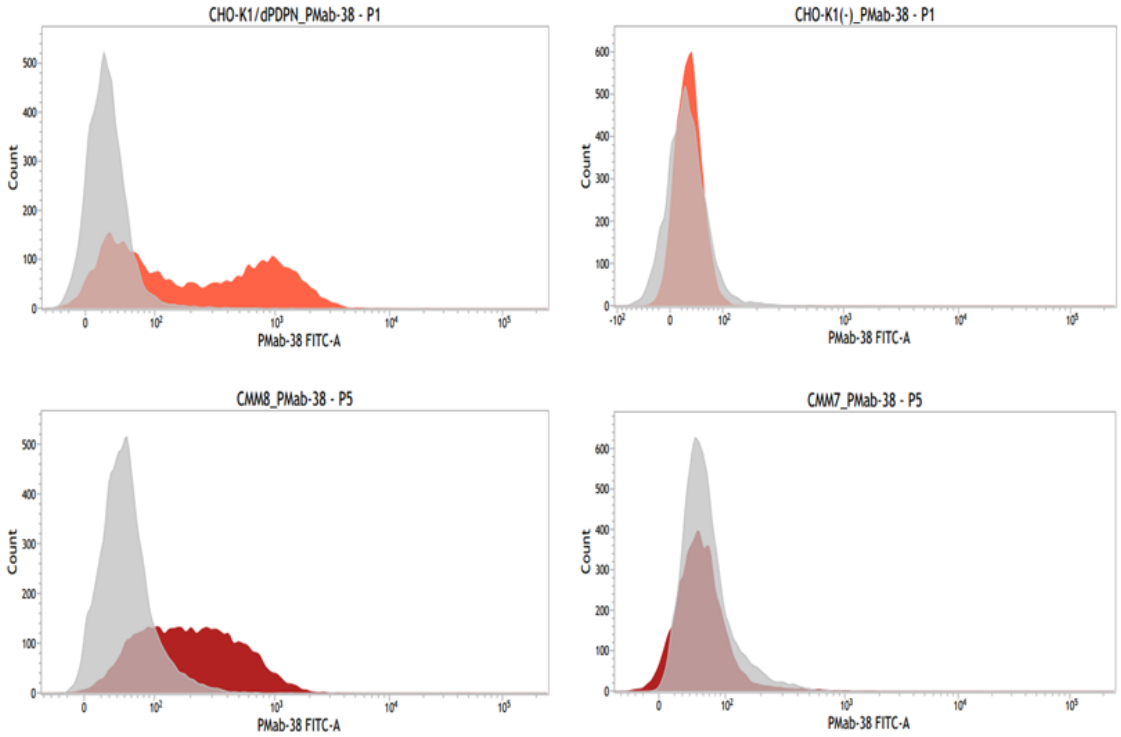

Fig. 3-2-3 PMab-38 detected PDPN on canine tumor cell lines

Representative staining of cell lines using PMab-38. After trypsinization of canine MM cell lines, cells were incubated with PMab-38 or purified mouse IgG1 as an isotype control, followed by wash in FACS buffer. Then, cells were incubated with anti-mouse IgG antibodies conjugated with FITC. Surface expression of PDPN were analyzed after live cells were gated by forward scatter/side scatter.

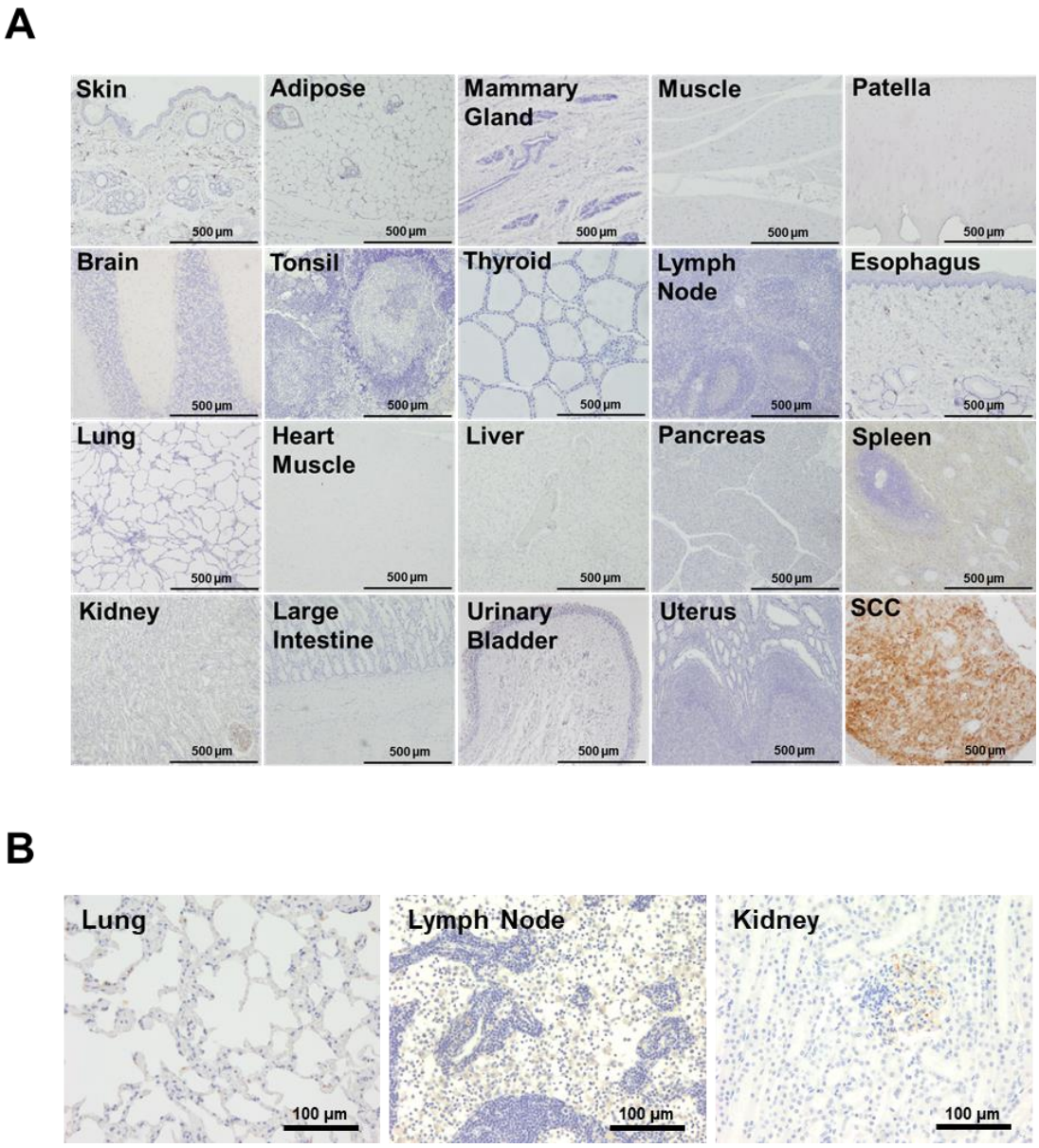

Fig. 3-2-4 Evaluation of PMab-38 staining in systemic normal tissues

(A) Representative staining pattern of IHC analysis using PMab-38 in systemic normal tissues. SCC specimen was used as a positive control for IHC analysis using PMab-38 staining. (B) Representative staining pattern of IHC analysis using PMab-38 in normal lung, lymph node, and kidney tissues at high-power field (x400).

Summary and Conclusion

Cancer is the one of the most common causes of disease-related death in humans, killing approximately 10 million people worldwide each year. On the other hand, the number of cancers in dogs has been increasing year by year and now accounts for a large percentage of canine deaths. Cancer treatments in general comprise three main modalities; surgery, chemotherapy and radiation therapy, but the efficacy of the combination of these three therapies are limited especially for patients with late stage cancer or cancer that is resistant to these therapies, which has been a longstanding problem in human and veterinary medicine. Thus, novel strategies are desperately required to treat cancer patients, and various approaches for the treatment of cancer patients have been developed. Among various approaches, immunotherapy approaches in cancer treatment have rapidly developed over the past decade, and immunotherapy is now the fourth pillar of cancer therapy in human medicine. The goals of cancer immunotherapy are to initiate or reinitiate a self-sustaining cancer immunity-cycle, enabling the cycle to amplify and propagate. To date, various types of cancer immunotherapies that act at different stages of this cycle to augment immune responses have been developed in both human and veterinary medicine.

Adoptive cell transfer (ACT), also known as cellular immunotherapy, is a technique in which cells are collected from a cancer patient, expanded and activated *ex*

vivo, and then transferred back into the patient in order to enhance the cancer immunity-cycle in cancer patients. Although LAK and TIL therapy have been developed, these therapies have not been widely applied in clinical practice because LAK cells are not able to recognize cancer cells and TILs are difficult to be adequately expanded *ex vivo* due to T cell exhaustion caused by tumor microenvironment.

In recent years, chimeric antigen receptor (CAR)-T cell therapy has been thought as the adoptive cell transfer that can overcome the challenges of LAK and TIL therapies. CARs are synthetic T cell receptors composed of an extracellular single-chain variable fragment (scFv) derived from the variable heavy and variable light domains of a given antibody, linked to transmembrane and intracellular signaling domains composed of the intracellular component of CD3z plus co-stimulatory molecules. T cells expressing the CAR on their surface, get activated following binding of the CAR to a specific antigen on the surface of cancer cells, leading to proliferation, expansion and effector function against the target cells. CD19 targeting CAR-T (CD19-CAR-T) cells have produced remarkable durable clinical responses in human patients with relapsed/refractory B cell malignancies, and CD19-CAR-T cells were approved for children with refractory and relapsed acute lymphoblastic leukemia (ALL) and adults with large B cell lymphoma (BCL) in 2017. Since the remarkable success with CD19-CAR-T therapy were reported, there has been much

research into CAR-T cell approaches to overcome some of the barriers to feasible and successful therapy and expand the types of tumor that can be treated using CAR-T cell therapy to effectively treat more cancer patients. Considering the remarkable success of CAR-T therapy in human medicine, CAR-T therapy may also be a promising treatment for cancer patients in veterinary medicine. In fact, CAR-T cell therapy has been explored in dogs for hematological and solid tumors *in vivo* and *in vitro* respectively. However, this approach is still in infancy in this species and more basic research is required to optimize this approach for use in canines with cancer.

The purpose of this study was to optimize CAR-T cell therapy for canine malignant tumors. In Chapter 1, CAR-T cells were generated from T cells from healthy dogs using a retroviral vector to obtain stable CAR expression on the surface of canine T cells, and antigen-specific effector function of the CAR-T cells against target cells was evaluated. In Chapter 2, the effect of different co-stimulatory domains on CAR-T cell function was compared to choose the most suitable CAR construct for clinical trials for canine patients with B cell lymphoma. Since fundamental information to generate canine CAR-T cells was revealed in Chapters 1 and 2, target antigens for CAR-T cells were explored to expand cancer types for further application of CAR-T cell therapy in dogs in Chapter 3.

In Chapter 1, retroviral transduction was performed for canine T cells to obtain stable CAR expression on the surface of canine T cells. Canine T cells were activated by CD3/CD28 magnetic beads on day 0, and two types of retroviral vectors (MigR1 and pMND) were used to transduce canine T cells with CAR on day 2. Then, canine T cells were cultured under the addition of IL-2 every other day, and the CAR expression on the surface of canine T cells was evaluated on day 8. On the same day, CAR-T cells were stimulated by adding irradiated antigen-expressing target cells and CAR expression were evaluated on day 16. In the results, surface expression of CAR last at least 16 days, and more than half of canine T cells expressed CAR on their surface on day 16. Then, to investigate antigen-specific effector function of CAR-T cells transduced by MigR1, CAR-T cell were co-cultured with target cells that do or do not express target antigens on day 8. In the results, the CAR-T cells showed cytotoxic activity, expression of degranulation markers, production of various cytokines (IFN-γ, TNF-α, IL-17) and proliferation antigen-specifically on day 8. These results suggested canine T cells were successfully transduced with CAR by retroviral vectors and the expression last at least on day 16, and the CAR-T cells transduced by MigR1 exhibited antigen-specific effector functions.

In Chapter 2, the effects of different co-stimulatory domains on the function of canine CAR-T cells were evaluated. First, four types of canine CAR were designed, a

first-generation CAR (no co-stimulatory domain) and second-generation CARs that contain one of three co-stimulatory domains; CD28, 4-1BB or ICOS. Then, canine T cells were transduced with each CAR construct in the same way as described in Chapter 1, and the cytotoxicity, expression of degranulation markers, cytokine production, and proliferation of the CAR-T cells were evaluated. As a result, the expression of cytotoxicity and degranulation markers tended to be higher in T cells containing first-generation CARs than in T cells containing CD28z, 4-1BBz and ICOSz-based CARs on day 8, but there was no clear difference in the cytotoxicity and the expression of degranulation marker among T cells containing different second-generation CARs. On the same day, CAR-T cells without co-stimulation domain, CD28z and 4-1BBz-based CAR-T cells produced three cytokines, IFN-γ, TNF-α and IL-17A, while ICOSz-based CAR-T cells produced almost none of these cytokines. CD28z-based CAR induced larger percentage of T cells to produce IFN-γ and/or TNF-α than other CAR constructs, but there was not clear difference in the cytokine production of IL-17A among the three types of CAR-T cells that showed cytokine production. Finally, CAR-T cells were stimulated with irradiated target cells, and proliferation of the CAR-T cells were compared on day 8 and day 16. As a result, CD28z-based CAR and 4-1BBz-based CAR maintained the proliferation of both $CD4^+$ and $CD8^+$ CAR-T cells until day 20, while first-generation

CAR and ICOSz-based CAR showed no or lower expansion of CAR-T cells by day 20. These results showed that there was no obvious difference in cytotoxicity and degranulation marker expression among all co-stimulatory domains, and that CD28z and 4-1BBz-based CAR-T cells were superior to ICOSz-based CAR-T cells in cytokine production and cell proliferation.

In Chapter 3, possible target antigens were explored in various solid tumors, to widen the range of tumor types that could be treated using CAR T cell therapy. Target antigens should be exclusively expressed on the surface of cancer cells to prevent on target, off-tumor side effects of therapy. On exception to this is if the target antigen is shared normal cell types that are nonessential. Considering the reports in human and the availability of cross-reactive antibodies for use in dogs, two antigens were chosen for further investigation; human epidermal growth factor receptor 2 (HER2) and podoplanin (PDPN). Studies designed to evaluate these two target antigens for CAR-T cell therapy of solid tumors are described in section 1 and 2 respectively.

In Section 1, HER2 expression was evaluated in canine anal sac adenocarcinoma (ASAC), primary lung cancer (PLC) and thyroid cancer (TC) by immunohistochemistry (IHC), chosen by a pre-screening experiment. In the results, HER2 expression was confirmed in canine ASAC (80%), PLC (69%) and TC (48%). In

addition, flow cytometry analysis using an anti-human HER2 antibody (trastuzumab), which was reported to have cross-reactivity to canine HER2, confirmed the expression of HER2 on the cell surface in canine mammary carcinoma (MAC), transitional epithelial carcinoma (TCC), osteosarcoma (OSA), and malignant melanoma (MM) cell lines. These results suggest that HER2-targeted CAR-T cell therapy may be applicable to anal sac adenocarcinoma, primary lung cancer and thyroid cancer, and that scFv derived from trastuzumab can be used for designing CAR construct.

In Section 2, PDPN was investigated as a possibility of target antigen for CAR-T cells. PDPN is a type I transmembrane sialoglycoprotein, which reported to be expressed in various tumor types in human such as squamous cell carcinoma (SCC). However, it has been thought to be difficult to use as a target antigen for CAR-T cells because it is also expressed in normal tissues. Recently, an anti-canine cancer-specific glycosylated PDPN antibody (PMab-38) has been developed that is thought to bind to abnormal glycosylated PDPN in cancer tissues. Firstly, the staining patterns of IHC were compared between PMab-38 and anti-pan-PDPN antibody to investigate the tumor specificity of this antibody. The results showed that the anti-pan-PDPN antibody showed positive images in alveolar epithelial cells, where PDPN is naturally expressed, whereas the PMab-38 did not. On the other hand, similar staining images were observed in SCC

tissue with both PMab-38 and anti-pan-PDPN antibody. Next, IHC analysis using PMab-38 was performed for 13 types of canine solid tumors and 19 types of normal tissues. The results showed that the positive staining of PMab-38 was frequently observed in cancer cells of SCC and PLC. The positive staining of PMab-38 was also observed in cancer-associated fibroblasts of SCC, ASAC and TCC. On the other hand, in normal tissues, a weak positive staining was observed in the kidney, but not in other 18 normal tissues including lymphatic vessels and type I alveolar cells, where PDPN expression has been reported. Finally, flow cytometry analysis confirmed that PMab-38 recognized PDPN on the cell surface in canine MM cell lines. These results suggest that aberrant glycosylated PDPN which recognized by PMab-38 may be a target antigen for CAR-T cell therapy in cancer types with positive staining of PMab-38, and that scFv derived from PMab-38 can be used for designing CAR construct.

These results indicate that stable CAR expression on canine T cells can be obtained by using retroviral vectors, and that insertion of co-stimulatory domains (CD28 and 4-1BB) into the CAR sustained the expansion of canine CAR-T cells. In addition, it was shown that HER2 and aberrant glycosylated PDPN would be candidates for target antigens for expansion of canine CAR-T cell therapy. Further studies are required to evaluate the anti-tumor effect of canine CAR-T cells *in vivo* and to verify their safety in dogs for clinical application.

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