博士論文

Development of new therapy for canine mammary cancer with a recombinant measles virus

(イヌ乳がんに対する組換え麻疹ウイルスを用いた新規治療法の開発)

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CONTENTS

SUMMARY

Oncolytic virus has been shown a hopeful strategy for cancer therapy. Our group previously showed that a recombinant measles virus (rMV-SLAMblind), which selectively used poliovirus receptor-related protein 4 (PVRL4) as a receptor and not signaling lymphocyte activation molecule (SLAM), had therapeutic effects against human breast cancer cells. Here, the author investigated applicability of the rMV-SLAMblind for treatment of canine mammary tumors. First, it was shown that rMV-SLAMblind could infect cells using canine PVRL4 as a receptor. The author found that canine PVRL4 was expressed in 4 of the 9 canine mammary cancer cells. Consistent with PVRL4 expression, rMV-SLAMblind efficiently infected the 4 PVRL4-positive cells, and achieved more than 70% cytotoxicity in two of them, CF33 and CHMm cells. *In vivo* assay, rMV-EGFP-SLAMblind suppressed significantly the progress of the tumor xenograted with CF33 cells in comparison with control group. Immunohistochemistry (IHC) examination revealed that PVRL4 was expressed in 45% of canine mammary tumors, and these tumor cells were efficiently infected with rMV-EGFP-SLAMblind. In normal dog tissues, canine PVRL4 was expressed in partially epithelial cells, skin, bronchi, tongue, renal pelvis and bladder. These tissues seemed to be susceptible to rMV-SLAMblind, however dogs inoculated with rMV-SLAMblind did not show any clinical symptoms in another study. The results showed that our rMV-SLAMblind was sufficiently safe for dogs. On the basis of these results, rMV-SLAMblind targeting canine PVRL4 could be a good

candidate for treatment of canine mammary tumors.

INTRODUCTION.

Cancer is an important cause of death. In veterinary region, the National Cancer Institute estimates that nearly 6 million new cases are diagnosed in dogs each year (Shilling et al., 2010). Canine mammary tumors (CMTs) are the most frequent neoplasms in female dogs. Some studies showed a diagnosed rate of approximately 200／100,000 dogs／year (Dobson et al. 2002; Merlo et al. 2008; Vascellari et al. 2009). More than 40% of tumors, within all tumors in female dogs, are CMTs (Dorn et al., 1968). CMTs are classified histologically into 4 subtypes. The most common subtype of CMTs is originated in epithelial cells (simple adenoma and simple adenocarcinoma). Some CMTs consist of both epithelial and myoepithelial (complex adenoma and complex adenocarcinoma). A few tumors are of mesenchymal origin (fibroadenoma, fibrosarcoma, osteosarcoma and other sarcomas). A combination of epithelial and mesenchymal tissues (mixed benign tumours and carcinosarcoma) is frequently observed (Hampe and Misdorp, 1974; Withrow et al., 2013; Misdorp et al., 1999; Misdorp, 2002). Fifty per cent of the CMTs are malignant and the majority of malignant CMTs in dogs are adenocarcinomas. Less than 5% are sarcomas (Hampe and Misdorp, 1974; Withrow et al., 2013).

Surgical resection is the standard treatment for CMTs, excluding the case of diagnosed dogs that have highly metastasis disease (Withrow et al., 2013; Misdorp, 2002). About 50% of dogs with malignant CMTs have complete remission with surgery alone (Misdorp and Hart, 1979; Straw, 2005). However, approximately half of dogs with malignant CMTs have usually metastatic cancer at

the time of surgery, which results in poor prognoses (Misdorp, 2002). In such cases, those diagnosed dogs are mainly treated with radiotherapy and chemotherapy.

In radiotherapy, several radiation protocols have already been provided for various canine tumors, such as nasal carcinoma, lymphoma and osteosarcoma. However, in the case of CMTs, radiotherapy has not yet been evaluated as a treatment option (Buchholz et al., 2009; Coomer et al., 2009; Deveau et al., 2010; Elliot and Mayer, 2009; Hunley et al., 2010; Lurie et al., 2009; Withrow et al., 2013; Andrew, 2003; Sorenmo, 2003). In chemotherapy, several compounds and molecular target therapies have been evaluated *in vitro* study and also in dogs with metastasized CMTs. However, most of the compounds showed high toxicity without any effectiveness (Clemente et al., 2009; Dominguez et al., 2009; De Souza et al., 2009; Karayannopoulou et al., 2001; Marconato et al., 2008; Poirier et al., 2004; Simon et al., 2006). As for molecular target therapies, anti-estrogen therapy, which was mostly used as hormonal therapies in human breast cancer, was tested in veterinary clinical trials (Craig, 1992; Morris et al., 1993; Yamashita et al., 2006). However, its antitumor activity was very low and caused estrogen-like side effects (vulvar swelling, vulvar discharge, behavioural changes, nesting and pyometra). Therefore, the use of anti-estrogen drug in dogs for CMTs was not recommended (Morris et al., 1993). As for another molecular target therapy, the effect of anti-ErbB-1 and anti-ErbB-2 antibodies, which were frequently used in human breast cancer treatment, was analyzed *in vitro* study.

The effect of these antibodies was lower in dog cells than in human cells, and they partly inhibited cell proliferation of canine tumor cells (Singer et al., 2012). Singer et al. (2012) considered that the difference of efficacy between dog and human cells was caused by antibody affinity and expression levels of those molecules in dog and human cells. Thus, effective treatments for CMTs except surgical resection have not been established, especially in the case of dogs with metastasis (Karayannopoulou et al., 2005; Philibert et al., 2003; Yamagami et al., 1996), and development of a new therapy for CMTs is required.

In recent years, preclinical researches and clinical trials of oncolytic virotherapy have been carried out to investigate their potency in the clinical settings (Bell and McFadden, 2014; Russell et al., 2012). In the veterinary field, several kinds of viruses, including adenovirus, reovirus, myxoma virus, vaccinia virus and canine distemper virus (CDV), were investigated as oncolytic agents (Alcayaga-Miranda et al., 2010; Gentschev, Stritzker and Hofmann et al., 2008; Gentschev et al., 2010; Gentschev et al., 2012; Hwang et al., 2013; Laborda et al., 2014; Patil et al., 2012a; Suter et al., 2005; Yazawa et al., 2003). Effect of oncolytic virtotherapy can be expected when virus infects and /or replicates in tumor cells more efficiently than in normal cells. Selective infection to tumor cells can be achieved by generally two different strategies. One is known in adenovirus, vaccinia virus and reovirus that the virus replication is regulated at transcription level and is dependent on the

interactions between tumor specific nuclear transcription factors and viral promoter/enhancer elements (Alcayaga-Miranda et al., 2010; Gentschev, Stritzker and Hofmann et al., 2008; Gentschev et al., 2010; Gentschev et al., 2012; Laborda et al., 2014; Patil et al., 2012a; Yazawa et al., 2003). However, those virus entries to normal cells are not able to be controlled, and thus virus replication in normal cells was sometimes observed (Patil et al, 2012b). On the other hand, the other strategy, which was taken for virotherapy with CDV, was based on specific molecules expressing on the surface of cancer cells. CDV efficiently infected canine lymphoma cells using signaling lymphocyte activation molecule (SLAM) as receptors (Suter et al., 2005). SLAM is expressed in lymphocytes and used by Morbilliviruses as the common receptor (Tatsuo et al., 2001). These results showed that Morbillivirus had a potential of oncolytic ability.

Measles virus (MV) belongs to the same genus Morbillivirus and family Paramyxoviridae as CDV. MV is an enveloped virus with a non-segmented single strand RNA genome. The viral genome of MV is approximately 16 kb in length and consists of six structural genes encoding the nucleocapsid (N), phosphor- (P), matrix (M), fusion (F), hemagglutinin (H) and large (L) proteins. Among these six structural proteins, H and F proteins are surface glycoproteins in the viral envelope. The H protein interacts directly with the cellular receptors and triggers the F protein to execute membrane fusion. When MV infected cells, H and F proteins are expressed on surface of infected cells

and induce syncytia, which amplified apoptotic signals as cytopathic effect (CPE), by interaction with cellular receptors of cells adjacent to infected cells (Griffin et al., 1996). Three cellular receptors for MV were identified. CD46 is a type I transmembrane protein, complement regulatory molecule and ubiquitously expressed in all human cells except erythrocytes. It was identified as a receptor of MV Edmonston vaccine strain (Dörig et al., 1993; Naniche et al., 1993). SLAM was identified as a receptor for both wild type and vaccine strain of MV (Tatsuo et al., 2000). SLAM is expressed on immune system; mature dendritic cells, macrophages, B cells, immature thymocytes and memory T cells, consistent with the lymphotropism of the wild type MV. Poliovirus receptor related protein 4 (PVRL4 also called nectin4) was identified as an epithelial cell receptor for both wild type and vaccine strain of MV (Mühlebach et al., 2011; Noyce et al., 2011). Its expression in normal human tissues is restricted to the placenta and to the trachea at slight level. Recombinant MVs, which were generated by reverse genetics system based on Edmonston strain, were reported as oncolytic virus in preclinical studies (Lech and Russell, 2010). Because CD46 is overexpressed in cancer cells, these oncolytic viruses efficiently infect cancer cells with targeting CD46 and show anti-tumor activity. The advantage of this therapy is that Edmonston strain is considered to be assured of safety because it has been utilized as MV vaccine for a long time (Griffin et al., 2008). However, the Edmonston strain can infect and spread to wide-ranging cells, because CD46 is ubiquitously expressed on all human cells

except erythrocytes (Liszewski et al, 1991). The carcinoembryonic antigen-expressing MV (MV-CEA) and human sodium iodide symporter-expressing MV (MV-NIS) based on Edmonstron strain were generated as the oncolytic agents to monitor virus spread noninvasively *in vivo,* and their oncolytic effects were evaluated in preclinical and in clinical situation (Peng et al., 2002; Dingli et al., 2004). These preclinical studies showed that MV should be promising candidates of oncolytic agent. Two patients, who were diagnosed relapsing drug-refractory myeloma and multiple glucose-avid plasmacytomas, were treated by the MV-NIS (Russell et al., 2014). While one patient achieved complete remission from all disease sites, the other patient's cancer had come back worse than before treatment. In this study, the amount of virus inoculated with both patients was around 10^{11} $TCID_{50}$ (50% tissue culture infectious dose). It was considered that the dose was much higher than that for vaccine use (10^4 $TCID_{50}$). Thus, the efficacy of the oncolytic MV should be further improved.

In previous study, Sugiyama et al. (2012) reported that a wild type MV HL strain had anti-tumor activity against human breast cancer cells. MV HL strain was isolated from leukocytes of a measles patient using marmoset lymphoblastoid B95a cells, (Kobune et al., 1996). Using a reverse genetics system, a recombinant MV that was unable to use SLAM (rMV-SLAMblind) was generated by introducing a single amino-acid substitution (R533A) into the open reading frame (ORF) of the H protein. The rMV-SLAMblind efficiently infected and killed human breast cancer cells as well as its

parental rMV. Because the pathogenicity of wild type MV is caused by infection to immune cells through SLAM and by spread to the entire body with infected immune cells, it is expected that the rMV-SLAMblind is not pathogenic including immunosuppression. Actually, rhesus monkeys, which were inoculated with the rMV-SLAMblind, did not show any clinical symptoms (Sugiyama et al., 2012; Leonard et al., 2010). On the other hands, rMV-SLAMblind remained efficient infection activity to human breast cancer cells through human PVRL4 (Sugiyama et al., 2012). In comparison with the oncolytic activity of Edmonston strain, that of rMV-SLAMblind was significantly higher in PVRL4-positive human breast cancer cells (Sugiyama et al., 2012). These results suggested that rMV-SLAMblind is more effective for breast cancer treatment and safer than oncolytic MV based on Edmonston strain. Therefore, it is considered that rMV-SLAMblind is a promising candidate as a novel oncolytic virus for breast cancer treatment.

PVRL4 gene encodes a member of Nectin family that belongs to the immunoglobulin superfamily, and it is classified into a type I transmembrane glycoprotein, which consists of three Ig-like ectodomains (V, and two C2 domains), a transmembrane region, and a cytoplasmic tail (Morrison et al., 1992). PVRL4 colocalizes with cadherins in adherens junctions. PVRL4 interacts with itself and associates with the actin-binding protein afadin and integrin αvβ3 (Reymond et al., 2001). While human PVRL4 was known to be normally expressed in the placenta and slightly in the

trachea, recent studies have shown that PVRL4 expression was detected also in human breast cancers. It was reported that the PVRL4 expression was detected in 62% ductal carcinoma type and 6% lobular carcinoma (Fabre-Lafay et al., 2007). The expression of PVRL4 positively related with the expression of the basal-like markers EGFR, P53, and P-cadherin, and negatively related with the expression of the luminal-like markers ER, PR and GATA3. In contrast, expression of PVRL4 was detected in many tumor cell lines which had luminal-like phenotypes. Although biological significance of PVRL4 expression remained to be revealed, Fabre-Lafay et al. (2007) considered that PVRL4 expression did not depend on the general classification of breast cancer. In addition to breast cancer, PVRL4 was expressed in lung, ovarian, pancreas and prostate cancer cells (Derycke et al., 2010; Takano et al., 2009). Furthermore, it was reported that increased PVRL4 expression rate also significantly related with size and malignancy of tumors (Athanassiadou et al., 2011). Moreover, Fabre-Lafay et al. (2005) has found that a soluble form of PVRL4 was detected in the sera of patients with metastatic breast cancer. The soluble PVRL4 is formed by the entire PVRL4 ectodomain and PVRL4 shedding is enhanced by ADAM metallopeptidase domain 17 (ADAM17) that is overexpressed in breast cancers (Fabre-Lafay et al., 2005). Actually, it was reported that concentration of soluble PVRL4 in sera correlated with cancer progression, and thus, it could be useful marker for clinical evaluation of patients with metastatic breast cancer (Fabre-Lafay et al., 2007). From these findings, PVRL4 has

been considered as a new tumor-associated antigen and therapeutic target of tumors.

MV, CDV, rinderpest virus (RPV) and Peste-des-petits-ruminants virus (PPRV) are classified in the same genus Morbillivirus. As for the interaction between PVRL4 and MV, it was reported that L482 and P497 in MV H protein were important for binding PVRL4 (Mateo et al., 2013). In contrast, there have been no reports about critical amino acid in PVRL4 for binding MV H protein. However, Delpeut et al. (2014) showed that CDV also infects cells through canine PVRL4 as an epithelial receptor, and the amino acid F132/P133/A134/G135 in canine PVRL4 were critical for binding CDV H protein. Because these domains were completely conserved in human PVRL4, and the critical amino acids motif (P493/Y539) of CDV H for interaction with canine PVRL4 was also found in MV H protein (P497/Y543), it was expected that MV H protein could also bind to those domain of canine PVRL4. However, there had not been analysis about whether MV is able to infect canine cells through canine PVRL4. MV has been studied using transgenic mice expressing functional receptor to study the interaction between viral components and antiviral factors of host cells, (Horvat et al., 1996). Welstead et al. (2005) generated human SLAM expressing mouse (hSLAM mouse) and inoculated these mice intranasally with wild type MV. However, MV infection was not detected in all tissues except in the nasopharyngeal lymph nodes, thus the model was not sufficient for the study. To improve the efficiency of MV infection, hSLAM mice were interbred with transgenic mice lacking a

signal transducer and activator of transcription 1 (Stat1). Stat1 acts as transcription activators via activation by cytokines such as interferone (IFN) and upregulates the expression of hundreds of genes concerning antiviral state (Schindler et al., 1992). These bred mice were a little more susceptible to MV infection and produced more virus particles. The result suggested that lack of antiviral activity was supprotive for efficient replication of MV in cells. IFN is one of the most important factors in antiviral factors. It is expected that MV is able to replicates efficiently in canine cancer cells.

In this study, the author investigated whether rMV-SLAMblind therapy can be applied to the treatment of canine mammary cancer (CMC).

MATERIALS AND METHODS

Cells

MCF7 was purchased from American Type Culture Collection (ATCC, Manassas, VA). Six CMC cells; CHMp, CHMm, CTBp, CTBm, CIPp and CIPm were kindly provided by T. Nakagawa (the University of Tokyo, Japan) and were grown in RPMI1640 (Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS), streptomycin (100 mg/mL) and penicillin (100 U/mL) (Uyama et al., 2006). Madin-Darby canine kidney (MDCK) cell was purchased from RIKEN BioResource Center (Tsukuba, Japan). CF33 was kindly provided by R.Asano (the University of Nihon, Japan). CBrC was kindly provided by K,Ogihara (unpublished). MDCK, CF33, CBrC and HEK293 cells were cultured in Dulbecco's modified minimum essential medium (DMEM; Life Technologies, Carlsbad, CA) containing 10% FBS and antibiotics. AZACB cells were purchased from COSMO BIO (Hokkaido, Japan) and cultured in Minimum Essential Medium Eagle (MEM; Sigma, St. Louis, Missouri) containing 10% FBS and 0.295% Tryptose Phosphate Broth (Sigma). All cells were cultured at 37 °C with 5% CO2.

Plasmid

The plasmids expressing canine SLAM or PVRL4 were generated as follows. The coding region of SLAM was amplified with Phusion High-Fidelity DNA Polymerase (New England Biolabs,

Ipswich, MA) using the following specific primers for canine SLAM (5'- GAA GAT CTG AAT GGA TTC CAG GGG CTT CCT- 3' and 5' – GCG TCG ACT CAG CTC TCT GGG AAC GTC A- 3'). The PCR product was cloned between BglII and SalI sites of pCAGGSneo vector, that is a pCAGGS vector possessing neomycin resistance gene. The coding region of PVRL4 was amplified with Phusion High-Fidelity DNA Polymerase using the following specific primers for canine PVRL4 (5'-AAG CTT GCC ACC ATG CCT CTA TCC CTG GGA GC-3' and 5'-GAA TTC TCA GCC CAT CAC AGA GCA GC- 3'). The PCR product was cloned into HindIII and EcoRI sites of pcDNA3.1 vector (Invitrogen, Carlsbad, California).

Transfectant cells

HEK293 cells were transfected with plasmid pCAGGS-neomycin canine SLAM or pcDNA3.1-canine PVRL4 respectively using lipofectamine LTX (Life Technologies) according to manufacturer's instructions. To select the cells expressing SLAM or PVRL4, the transfected cells were cultured with medium containing 0.5-1 mg/mL of G418 for 3 weeks. The expression of canine SLAM and canine PVRL4 were confirmed by reverse transcription polymerase chain reaction (RT-PCR). These transfectant cells were named as HEK293/canineSLAM or HEK293/caninePVRL4, respectively.

Virus

rMV-EGFP and rMV-EGFP-SLAMblind were propagated in MCF7 cells as previously described (Sugiyama et al., 2012). Virus titers were determined as 50% tissue culture infectious dose ($TCID_{50}$) by the Reed–Muench method (Reed and Muench, 1938) and titrated using CF33 cells.

RT-PCR

Total RNA was extracted from cells using ISOGEN (Nippon Gene, Tokyo, Japan) according to manufacturer's instruction. Reverse transcription (RT) was performed by using PrimeScript RTase (Takara, Otsu, Japan). PCR amplifications were performed by using Amplitaq Gold (Life technologies). The canine SLAM, canine PVRL4 or hypoxanthine guanine phosphoribosyl transferase (HPRT) genes were amplified using the following primers; canine SLAM forward, 5'- TCA TGA CCC TGG AGG AGA AC-3'; canine SLAM reverse, 5'- GGT CAA TCC CCA GTT TCT CA-3'; canine PVRL4 forward, 5'- GTC ACT TCG GAG TTC CAC CT-3'; canine PVRL4 reverse, 5'- TGA GTG TAG CGC CTT CTC TG-3'; HPRT forward, 5'- TGC TCG AGA TGT GAT GAA GG-3'; HPRT reverse, 5'- TCC CCT GTT GAC TGG TCA TT-3'.

Infection assay in transfectant cells

5.0×10^5 of HEK293, HEK293/canineSLAM and HEK293/caninePVRL4 cells were seeded in 12-well plates. After incubation at 37 °C for 1 hour, cells were infected with rMV-EGFP or rMV-SLAMblind at a multiplicity of infection (MOI) of 0.01. Virus infection was detected by EGFP under a confocal laser scanning biological microscope FV-1500 (Olympus Optical Co., Tokyo, Japan). Image J 1.48V (National Institutes of Health, Bethesda, MD) was used for calculating the degree of infected cells.

Infection inhibition assay using antibodies

HEK293/caninePVRL4 cells were seeded in 96-well plate and pretreated for 1 hour at 37 °C with medium containing 10 μg/mL of anti-Human Nectin-4 Affinity Purified goat Polyclonal Antibody (anti-PVRL4 antibody, R&D systems, Minneapolis, MN,) or goat control IgG (control IgG, R&D systems). Cells were infected with rMV-EGFP-SLAMblind at a MOI of 0.01. Cells were observed under a confocal microscope at 48 hours post infection.

Growth kinetics

CF33 cells were infected with rMV-EGFP-SLAMblind at a MOI of 0.01 and incubated

DMEM supplemented with 0.5% FBS. At every 24 hour, cell-free virus was obtained from the culture supernatants, and cell-associated virus was harvested by three freeze-thaw cycles. Virus titers were determined using CF33 cells.

Cell viabilities

HEK293, HEK293/canineSLAM and HEK293/caninePVRL4 cells were infected with rMV-SLAMblind at a MOI of 0.1. Regarding CF33, CTBm, and CHMm cells, they were infected with rMV-EGFP-SLAMblind at a MOI of 2. Cell viability were determined at every 48 hour by Premix WST-1 Cell Proliferation Assay System (Takara, Otsu, Japan) and by absorbance at 450 nm on a Microplate reader model 450 (Bio-Rad, Hercules, CA). The viability of cells infected with virus was calculated as the mean of absorbance values divided by the mean of absorbance values of uninfected cells and was expressed as a percentage.

Assessment of *in vivo* oncolytic activity

All Animal experiments were approved by the Experimental Animal Committee of The University of Tokyo.

Six-week-old female severe combined immune deficiency mice (C.B-17/Icr-scid/scidJc1)

were purchased from Clea Japan (Tokyo, Japan). CF33 cells (5×10^6) were suspended in 50 μL Hank's Balanced Salt Solution (HBSS; Life Technologies) containing 2% FBS and the suspension was mixed with 50 μL of Matrigel (BD Biosciences, San Diego, CA). The mixture was injected into the flank of mouse. At 9 days post implantation, mice were administered intratumorally with 10^6 $TCID_{50}$ of rMV-EGFP-SLAMblind (n = 8) or Opti-MEM (Life Technologies) (n = 8). Virus inoculation was repeated at 7 days after first inoculation. Tumor diameters were measured with calipers at every 2 or 3 days until 50 days post first inoculation. Tumor volume was calculated based on the formula (width × width × length)/2. All mice were euthanized at 50 days after first virus inoculation and tumor samples were collected. Some mice were euthanized at 4 days after first inoculation and tumor samples were collected.

Pathological Analysis

The collected samples from xenografts were fixed with 4% Paraformaldehyde Phosphate Buffer Solution (PFA, Wako, Osaka, Japan). After dehydration with concentration gradient sucrose, samples were frozen with OCT compound (Sakura Finetek, Tokyo, Japan) and sections were made a thickness of 5 μm on a cryostat (Leica CM1900, Leica Microsystems, Wetzlar, Germany). The nucleus was stained with 2 μg Hoechst33342 (Cambrex, New Jersey). EGFP deprived from virus

infection and Hoechst33342 were observed with a confocal microscope.

Immunohistochemical analysis (IHC) was performed in 5 μm sections from frozen tissue or 3 μm paraffin section. Frozen sections were fixed in acetone for 5 minutes at -20 °C. Paraffin sections were deparaffinized in xylene, rehydrated in graded ethanol and washed in PBS. Antigen retrieval was conducted for 15 minutes with autoclave in 0.01 M citrate buffer (pH 6.0). Frozen and Paraffin sections were incubated in 3% H_2O_2 in Phosphate buffered saline (PBS) for 10 minutes to quench encanineenous peroxidase activity. After washed in PBS, Slides were incubated in 2.5% normal horse serum for 20 minutes to block unspecific reaction and were stained with 2 μg anti-PVRL4 antibody or 2 μg control IgG as primary antibody at 4 °C overnight. After washed in PBS for 15 minutes, slides were incubated with ImmPRESS Reagent, Anti-Goat Ig (Vector laboratories, Burlingame, CA) for 30 minutes at room temperature. After washed in PBS for 15 minutes, slides were visualized with 3,3'-diaminobenzidine (DAB) and haematoxylin as counterstain.

Flow cytometry

Cells (1×10^6) were stained with 0.2 μg primary antibodies in 100 μL of sample buffer (PBS with 2% FBS and 0.02% NaN_3) on ice for 45 minutes. The following primary antibodies were used: anti-PVRL4 antibody and control IgG. Cells were washed once and stained with 0.1 μg of Alexa

Fluor 488 rabbit anti-goat IgG (Molecular Probes, Eugene, OR) in 100 μL of sample buffer on ice for 45 minutes. After wash, cells were resuspended with PBS containing 7-Amino- ActinomycinD (7-AAD, Beckman Coulter Immunotech, Massielle, France). Flow cytometry analysis was perfomed using a BD FACSCalibur or BD FACSVerse (BD Biosceinces, San Diego, CA), and obtained data were analyzed with Flowjo software ver 9.7.5 (TreeStar, San Carlos, CA).

Primary mammary tumor cell culture

Tumor samples were obtained by clinical surgery at animal hospital. Solid tumor sample was digested with HBSS supplemented with 1 mg/mL collagenase (Wako) and 0.1% DNase at 37 °C for 2 hours. After the cells were washed twice with HBSS containing 2% FBS, they were analyzed by flow cytometry, followed by culture with DMEM containing 10% FBS in 6-well plate. Primary tumor cells were infected with rMV-EGFP-SLAMblind at a MOI of 0.01.

Real time RT-PCR

Each tissue was homogenized with 1 mL ISOGEN using a Beads Crusher (Taitec, Saitama, Japan). After centrifugation, RNA was extracted from the supernatant according to manufacturer's instruction. The total RNA (1 μg) was reverse transcribed into cDNA using PrimeScript RTase

(Takara) with random hexamer primers. Real-time PCR was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) with Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan). HPRT was used as an internal control. The same primers of PVRL4 and HPRT as RT-PCR were used for real time PCR. Calculation of the cycle threshold value (CT) was based on automatic adaptive baseline settings.

Fluorescent antibody method (FA)

Paraffin sections were prepared by the same method as IHC. Slides were stained with 2 μg anti-PVRL4 antibody or 2 μg control IgG as primary antibody at 4 °C overnight. After washed in PBS for 15 minutes, slides were incubated with Alexa Fluor 488 rabbit anti-goat IgG (Molecular Probes) and Hoechst33342 for 45 minutes at room temperature. Images were observed with a confocal microscope FV-1500.

Statistical analysis

Statistical analysis of *in vitro* studies were performed by Student *t* test or one-way ANOVA with the Tukey test, and that of *in vivo* studies were was performed by Welch's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

Receptor usage of rMV-EGFP-SLAMblind

First, the author examined whether rMV-SLAMblind infects cells using canine PVRL4, but not canine SLAM. The author established canine PVRL4 or canine SLAM expressing HEK293 cells (HEK293/caninePVRL4 or canineSLAM) (Fig. 1A). To monitor virus infection, rMV-SLAMblind expressing enhanced green fluorescent protein (rMV-EGFP-SLAMblind) was used, because it was reported that replication ability was not influenced by EGFP insertion (Terao-Muto et al., 2008). Cells were inoculated with rMV-EGFP-SLAMblind at a MOI of 0.1 (Fig. 1B). Within 2 days post infection (dpi), rMV-EGFP-SLAMblind efficiently infected HEK293/caninePVRL4 cells and induced syncytia but not infected HEK293/canineSLAM cells. rMV-EGFP-SLAMblind hardly infected the parental HEK293 cells (Fig. 1B, C). To confirm whether canine SLAM of HEK293/canineSLAM cells was functional and usage of canine PVRL4 was the common characteristic of wild type MV, these cells were inoculated with the rMV-EGFP. rMV-EGFP infected efficiently and induced syncytia in both HEK293/canineSLAM cells and HEK293/caninePVRL4 cells (Fig. 1B, C).

To ascertain that rMV-SLAMblind infected HEK293/caninePVRL4 cells via canine PVRL4, cells were pretreated with anti-PVRL4 antibody followed by inoculation with rMV-SLAMblind. The number of infected cells and syncytia formation in HEK293/caninePVRL4 were dramatically reduced by anti-PVRL4 antibody treatment (Fig. 1D, E). These results suggested

that rMV- SLAMblind only uses canine PVRL4 as a receptor, while wild type MV possesses potential to use both canine SLAM and PVRL4 as receptors.

PVRL4 expression on CMC cells

To examine whether canine PVRL4 is expressed in CMT cells, nine CMC cell lines were analyzed by flow cytometry (Table 1). Four of nine CMC cells; CF33, CHMm, CTBp and CTBm cells expressed canine PVRL4 (Fig. 2A, B). CHMp/m, CTBp/m and CIPp/m cells were derived from three different dogs. CHMp, CTBp and CIPp were established from primary lesions and CHMm, CTBm and CIPm cells were established from metastatic lesions. Interestingly, CHMp cells did not express canine PVRL4 but CHMm expressed one. CTBm cells also expressed PVRL4 (Fig. 2B). The results indicated that canine PVRL4 expressed in about a half of CMC cells and expressed not only in primary lesion but also in metastasis lesion.

Infectivity of rMV-EGFP-SLAMblind to CMC cells and replication.

To examine infectivity of rMV-SLAMblind to CMC cells, CMC cells were inoculated with rMV-EGFP-SLAMblind at a MOI of 2. Although CBrC, AZACB, CHMp, CIPp and CIPm cells that didn't express canine PVRL4 were hardly infected, CF33, CHMm, CTBp and CTBm cells expressing

canine PVRL4 were efficiently infected (Fig. 3A). To analyze whether rMV-EGFP-SLAMblind was able to replicate well in canine cells, CF33 cells were inoculated with rMV-EGFP-SLAMblind at a MOI of 0.01. Cell-free virus and cell-associated virus were harvested and determined titers by a standard method. Virus grew well until 5 dpi and both the cell free and cell-associated virus titer peaked at 5 dpi (Fig. 3B, C). These results demonstrated that rMV-EGFP-SLAMblind efficiently infected and replicated in canine tumor cells.

***In vitro* cytotoxicity of rMV-SLAMblind depends on PVRL4 expression**

To observe the specific cytotoxicity against PVRL4 expressing cells, HEK293 cells, HEK293/canineSLAM cells and 293/caninePVRL4 cells were infected with rMV-EGFP-SLAMblind. Although rMV-EGFP-SLAMblind did not exhibit cytotoxicity against HEK293 cells and HEK293/canineSLAM cells, the viability of HEK293/caninePVRL4 cells were dramatically declined over 4 days (Fig. 4A).

Three CMC cells expressing PVRL4 were also infected with rMV-EGFP-SLAMblind and were measured their cell viabilities. rMV-EGFP-SLAMblind killed 71% of CF33, 56% of CTBm and 68% of CHMm at 7 dpi (Fig. 4B). The viability of CTBp cells at 7 dpi was unable to be analyzed because the cells were weak and cast off in the 7 days culture. These results indicated that

rMV-EGFP-SLAMblind had anti-tumor activity *in vitro* by the specific infection using PVRL4.

Oncolytic activity of rMV-SLAMblind in CF33 xenograft model

To assess the oncolytic ability of rMV-SLAMblind *in vivo*, CF33 cells were transplanted into SCID mice. The xenograft mice were inoculated intratumorally with 10^6 $TCID_{50}$ of rMV-EGFP-SLAMblind and the virus was inoculated again at 7 days post first inoculation. Tumor size was measured at every 2 or 3 days. Although the tumor in the control group grew larger, virus inoculation group exhibited the significant suppression of tumor growth (Fig. 5A). At 50 days post first inoculation, the mice were euthanized and performed autopsy. The tumor size of mice which were inoculated with rMV-EGFP-SLAMblind was obviously smaller than that of control mice (Fig. 5B, C). The expression of canine PVRL4 of transplanted CF33 cells was confirmed by IHC (Fig. 5D). To observe virus infection in tumors, some mice inoculated with rMV-EGFP-SLAMblind were euthanized at 4 dpi. EGFP were observed in many tumor cells and syncytia were observed with a high magnification (Fig. 5E). These results suggested that rMV-SLAMblind replicated well also *in vivo* and showed anti-tumor activity with forming cell-to-cell fusion.

Analysis of PVRL4 expression in CMT tissues and primary infection *in vitro*

So far, we demonstrated that PVRL4 was often expressed on CMC cell lines. However, it is important to find how frequently PVRL4 is expressed in clinical cases of CMTs, in order to expect applicability of virotherapy with rMV-SLAMblind. Therefore, we analyzed the expression of canine PVRL4 using clinical tumor tissues by IHC or FA comparing that in normal mammary tissues as basal level of PVRL4 expression. While PVRL4 positive signals were detected in sebaceous gland, canine PVRL4 was not detected in normal mammary tissues, including epithelial and myoepithelial cells (Fig. 6A). On the other hand, among eleven tumor tissues, canine PVRL4 was detected in two malignant, two benign and one unclassified tumor tissues (Table 2, Fig. 6B, C). To analyze infectivity in clinical isolated tumor, primary cells derived from clinical isolated tumor of case No.2 were inoculated with rMV-EGFP-SLAMblind. Approximately half of cells were efficiently infected to be killed (Fig. 6D). To confirm the ratio of PVRL4 expressing-cell population in these primary cells, PVRL4 expression of cells was analyzed by flow cytometry. Canine PVRL4 was detected in 46% cells of viable cells with main population based on FSC and SSC (Fig. 6E). The result that the ratio of PVRL4 expressing-cell population was similar to that of infected-cell, suggested rMV-SLAMblind infects PVRL4-positive cells specifically. These results suggested that canine PVRL4 is expectedly expressed in nearly half of clinical tumor samples of CMTs, and rMV-SLAMblind has ability to kill the tumor cells expressing PVRL4.

Expression of PVRL4 in normal canine tissues

Virus tropism is related with expression of a viral receptor molecule. Considering side effects of rMV-SLAMblind therapy, it is important to understand expression pattern of PVRL4 in whole body of normal dogs. First, the author analyzed expression of PVRL4 mRNA in 26 tissues by real time RT-PCR (Fig. 7A). PVRL4 mRNA was detected in lung, kidney, pancreas, tongue, esophagus, stomach, skin, bladder and uterus. It was reported that PVRL4 was detected in brain and intestine by IHC (Pratakpiriya et al., 2012), but expression level of PVRL4 mRNA was lower than detection limit in this experiment. To examine whether PVRL4 protein is expressed in the tissues in which PVRL4 mRNA was detected, the author generated paraffin sections of these tissues and performed IHC or FA. PVRL4 was expressed in location of prickel cell layer and basal cell layer in tongue and esophagus (Fig. 7B, C). In renal pelvis and bladder, the expression was detected in the entire cells of epithelial tissues (Fig. 7D, E). In lung, PVRL4 was localized in bronchial epithelium (Fig. 7F, G). In skin, it was detected in epidermal cells and hair follicle cells (Fig. 7H, I, J). Moreover, in fat pad and oral epithelium the expression was also observed (Fig. 7. K, L, M, N). These results indicated that PVRL4 was localized in epithelial cells, especially stratified squamous epithelium tissue and transitional epithelium tissue.

DISUCUSSION

In this study, the author investigated whether rMV-SLAMblind could be a candidate as novel oncolytic virus for CMT treatment. The author showed that rMV-SLAMblind effectively infected CMC cells, and showed anti-tumor activity to the cells expressing canine PVRL4 *in vitro* and in xenografts.

In the veterinary field, the treatment for metastasis of CMTs is practiced using classic chemotherapy drugs following with strong side effects, and there were a little evidence for efficacy of molecular target therapy such as anti-estrogen therapy or epidermal growth factor receptor (EGFR) / human epidermal growth factor receptor (HER2) kinase inhibitors which were reported to be effective for human breast cancer (Craig, 1992; Smith et al., 2007; Yamashita et al., 2006). However, efficacy of them was limited. Therefore, it is necessary to develop an effective treatment for metastasis cancer of CMTs. In this thesis, the author showed that canine PVRL4 was expressed in CHMm and CTBm cells derived from metastasis lesions, and rMV-SLAMblind showed cytotoxicity for these cells. In particular, CHMm cells expressed canine PVRL4, while the CHMp cells derived from its' primary lesion did not. The result suggested that canine PVRL4 was possibly expressed in highly malignant mammary cancer. This was supported with the results of IHC analysis of clinical tissues, which showed canine PVRL4 was expressed in 29% of benign tumor samples but expressed in 67% of malignant cancers. Though the number of samples was small in this study, expression of canine

PVRL4 seemed to be correlated with malignancy of CMT. The rMV-SLAMblind was expected to be a novel candidate for treatment of CMTs, particularly of malignant tumors that were not responsive to anticancer drugs.

Flow cytometry analysis of primary CMT cells indicated that the number of PVRL4 expressing cells were below the half of total tumor cells. Thus, the CMT cells were considered to have heterogeneous population. In this analysis, the primary CMT cells from tissues of diagnosed dogs included non-tumor cells such as fibroblasts. This heterogeneity makes it difficult to decide a prognosis and treatments for CMT dogs, as is often occurred in human cases (Nieto et al., 2000; Shipitsin et al., 2007). The rMV-SLAMblind therapy is effective only for PVRL4 expressing cell population, however, the therapy seems to be available to the dogs who are diagnosed as poor prognosis. Further, this therapy can be one of the choices, which are adopted before surgical resection or chemotherapy.

In this study, it was shown that wild type MV was able to infect cells using canine PVRL4 as a receptor. Because MV can utilize canine SLAM as a receptor, it is no wonder that MV shows pathogenicity in infected dogs (Tatsuo et al., 2001). However, there were no reports that dogs contracted MV, and actually the author found that rMV-SLAMblind could not efficiently infect PVRL4 expressing MDCK cells. These results suggested that virus entry alone was not sufficient to

ensure effective replication of the MV in the non-natural host. In contrast, all of canine cancer cells expressing PVRL4 used in this study were susceptible to rMV-SLAMblind infection and replication. The result indicated that those cancer cells had required factors for MV replication. The Newcastle disease virus (NDV), which belongs to the Avulavirus genus in the family Paramyxoviridae, was reported to have oncolytic capacity (Alexander et al., 1988). NDV causes respiratory, digestive and neurological symptoms in birds, but human is not susceptible to NDV. However, it was reported that NDV could efficiently replicate and show anti-tumor activity in human tumor cells (Nelson, 1999; Pecora et al., 2002; Schirrmacher et al., 1999). Replication of NDV was inhibited by antiviral cytokines such as IFN in normal cells, but not in cancer cells that failed to develop an appropriate antiviral state (Krishnamurthy et al., 2006; Lam et al., 2011; Lech and Russell, 2010). It was reported that wild type MV also has infectivity and replicates effectively in the non-natural host cells in the presence of antagonists of antiviral host protein (Iwasaki et al., 2011). Considering these reports, rMV-SLAMblind should effectively replicate in cancer cells but not in normal cells.

Canine PVRL4 was reported to be expressed in epithelial cells of some dog tissues (Pratakpiriya et al., 2012). In addition, the author showed that canine PVRL4 was expressed in skin, tongue, esophagus, fat pad and oral epithelium. Our group studied whether rMV-SLAMblind could infect normal dog tissues expressing PVRL4 and cause pathogenicity. The results showed that all dogs

with rMV-SLAMblind inoculation did not show any clinical symptoms, and the viral RNA was not detected in any tissues even which expressed PVRL4 (data not shown). The reason seemed to be explained by the infection routes of MV. Wild type MV particles primary infect resident SLAM expressing alveolar macrophages (AMs) or dendritic cells (DCs) in the airway lumen or just below the epithelial cell layer. MV infected immune cells then migrate to lymph node and MV infection spreads to resident T and B lymphocytes, which amplify the virus and cause primary viremia. Following dissemination of the amplified virus to secondary lymphoid organs, infection of the primary airway epithelial cells occurs via PVRL4 of the basolateral surface, presumably through contact with infected primary immune cells (De Swart et al., 2007; Noyce et al., 2012). Because our rMV-SLAMblind was generated to lose SLAM binding capacity, the virus should not be able to spread to epithelial cells expressing PVRL4. Therefore, the rMV-SLAMblind is expected not to cause side effects for normal dogs even if the canine PVRL4 is expressed in some normal tissues.

In the previous study, Suter et al (2005) reported that CDV infection led to apoptotic cell death and could be a candidate for oncolytic virus. Because CDV also infects cells using canine PVRL4 as a receptor, the author generated a recombinant CDV that lost ability to use canine SLAM (rCDV-SLAMblind) as an oncolytic agent. The rCDV-SLAMblind was successfully rescued, but its replication in canine tumor cells was not sufficient (data not shown). In contrast, the rMV-SLAMblind

grew well not only in human, but also in canine cells. Thus, the author proceeded to investigate rMV-SLAMblind as an oncolytic agent in this study. Moreover, utilization of rMV-SLAMblind is seemed to have some more advantage than rCDV-SLAMblind. A major impediment to systemic application of oncolytic virus is pre- and post-existing neutralizing antibody (Bell and McFadden, 2014; Russell et al., 2012). Pre-existing neutralizing antibody is existed in sera, because the most dogs are vaccinated against CDV. The author confirmed that cross reactivity was lower than detectable level between rMV-SLAMblind and CDV wild type strain. This result agreed with the report of Orvell et al (1974). Further, the rMV-SLAMblind showed effective infection to cancer cells even in the presence of serum from dogs vaccinated with CDV (data not shown). When the therapy using rMV-SLAMblind will be applied to the CMTs that were spread by metastasis, it shall be inoculated intravenously. Therefore, it is important not to be blocked by serum antibodies. After rMV-SLAMblind reaches tumor cells, it spreads in tumor with forming cell-to-cell fusion. MV infection causes T-lymphocyte-mediated immune responses, and MV-specific CD8 T cells were activated and expanded during MV infection (Hickman et al., 1997; Jaye et al., 1998; Permar et al., 2003; Van Binnendijk et al., 1989; Ward et al., 1990). Galanis et al (2014) showed that MV treatment against human ovarian cancer triggered cellular immunity against the patients' tumor, which supported an immune mechanism in mediating antitumor effects. Thus, rMV-SLAMblind treatment may also

activate tumor antigen-specific T cells, and the immune reaction should be helpful for the therapy. Immune reactions after the rMV-SLAMblind therapy should be further studied.

It was reported that many oncolytic viruses are effectiveness for cancer treatment in preclinical situation. However, there were a few oncolytic viruses that showed anti-tumor activity in clinical situation. The cause of the results is considered that many oncolytic viruses were evaluated by using xenograft mouse model, which transplant clonal tumor cell lines into the immune deficient mouse. It is important for new agents to examine the anti-tumor effect agaisnt xenograft model. However, this assay has two problems. One is that immune system is deficient. The other is that tumor in mice is clonal population. Then recently it is recommended to use suitable model that have intact immune systems and have spontaneous tumor. Thus, we focused on veterinary field. In this study, the author indicated that rMV-SLAMblind therapy for CMCs has anti-tumor effects as well as for human breast cancer. CMTs can provide a suitable natural model for the comparative study of human breast cancer. Moreover, CMTs occur at a frequency of 3 times the incidence of mammary tumors in humans (Priester et al., 1971). The clinical trials in veterinary field are expected to obtain more information about the immune reaction and the kinetics of rMV-SLAMblind therapy. The information is helpful not only for CMC therapy but also for human breast cancer therapy.

In conclusion, the author showed that the rMV-SLAMblind efficiently infected canine

mammary cells through canine PVRL4 in cell culture and in xenografts. In addition, the author demonstrated that canine PVRL4 was expressed in clinical CMT tissues, nevertheless it was not detected in normal mammary tissue. These data indicated that rMV-SLAMblind targeting canine PVRL4 would be a hopeful candidate for CMT therapy that express PVRL4.

FIGURE LEGENDS

Figure 1. Receptor usage of rMV-EGFP-SLAMblind (**A**) HEK293/canineSLAM and HEK293/caninePVRL4 cells were generated and PVRL4 expression was confirmed by RT-PCR. (**B and C**) Cells were infected with rMV-EGFP and rMV-EGFP-SLAMblind at a MOI of 0.01. (**B**) Cells were photographed at 2 dpi. Magnification: ×100. (**C**) Area of EGFP fluorescene of a visual field was quantified using Image J1.48V, with integrating at five random fields in (B). Data represent means ±SD of three independent experiments. $^{*}P < 0.05$, when compared rMV-EGFP. N.S. means not significant. (**D and E**) HEK293/caninePVRL4 was infected with rMV-SLAMblind at a MOI of 0.01 in the presence of 10 μg/mL PVRL4 antibody or control IgG. (**D**) Photograph was taken at 2 dpi. Magnification ×100. (**E**) Area of EGFP fluorescene of a visual field was quantified using Image J1.48V, with integrating at five random fields in (D). Data represent means ±SD of three individual experiments. $^{*}P < 0.05$, when compared control IgG.

Figure 2. PVRL4 expression on canine mammary tumor cell lines. (**A and B**) Cell surface expression of canine PVRL4 on canine mammary tumor cells was analyzed by flow cytometry. Cells incubated with anti-PVRL4 antibody (white histogram) or control IgG (gray histogram) followed by incubation with Alexa488-conjugated rabbit anti-goat antibody. Data was analyzed with flowjo.

Figure 3. Infection of rMV-SLAMblind to CMT cells and replication of that in a CMC cell. (**A**) Cells derived from CMTs were infected with rMV-EGFP-SLAMblind at a MOI of 2. At 2 dpi, cells were observed under a fluorescence microscope. Magnification: ×100. (**B**) CF33 cells were infected with rMV-SLAMblind at a MOI of 0.01. Infection titers of cell-free virus and cell-associated virus were determined at various time points. (**C**) The photos show expansion of rMV-SLAMblind in CF33 at every 24 hours.

Figure 4. *In vitro* cytotoxicity of rMV-SLAMblind. (**A**) HEK293, HEK293/canineSLAM and HEK293/caninePVRL4 cells were infected with rMV-SLAMblind at a MOI of 0.1. Cell viability was measured every 24 hours by WST-1 assay. Data represent means ±SD in three independent experiments. * ,†; $p < 0.05$ and **, ††; $p < 0.01$ by Tukey's test compared cell viability of HEK293 and HEK293/dSLAM cells. (**B**) Three canine cancer cells, CF33, CTBm and CHMm, were infected with rMV-SLAMblind at a MOI of 2. Cell viability was measured at every 48 hours by WST-1 assay. Data represent means ±SD in three independent experiments.

Figure 5. Anti-tumor activity of rMV-EGFP-SLAMblind in xenografts. (**A**) CF33 tumor was established in SCID mouse. Mice were inoculated intratumorally with either rMV-EGFP-SLAMblind

at a doze of 10^6 $TCID_{50}$ or HBSS as control. Viruses were inoculated at day 0 and 7 (arrow heads). Welch's *t* test was used to compare two groups. Error bars represent ±SD. $^*P < 0.05$ was considered as statistically significant $^*P < 0.05$, $^{**}P < 0.01$. (**B**) At 50 days post the first inoculation, tumor samples were collected. Upper samples were collected from control group and lower samples from group with virus therapy. (**C**) The weight of the tumors collected at day 50 was measured. Data represent means ± SD. $^*P < 0.05$ was considered as statistically significant by Welch's *t* test. **(D)** At 4 dpi, frozen sections were prepared and stained with control goat IgG (left panel) or anti-PVRL4 antibody (right panel) as primary antibody. ImmPRESS Reagent anti goat IgG and 3,3'-diaminobenzidine (DAB) were used to visualize. Sections were counterstained with Hematoxylin. Bar: 50 μm. Magnification: ×100 (**E**) At 4 dpi, frozen sections were prepared and stained by Hoechst33342. EGFP and nucleus were observed under a confocal microscope. Magnification of left panel was ×100 and right panel was ×600.

Figure 6. Expression of PVRL4 in normal mammary tissue and in CMT clinical tissues. (**A**) 3 μm paraffin section of normal mammary tissue was stained with control IgG (left panel) or anti-PVRL4 antibody (right panel) as primary antibody. ImmPRESS Reagent anti goat IgG and DAB were used to visualize. Sections were counterstained with Hematoxylin. Asterisk indicates sebaceous

gland. Bar: 100 μm. Magnification: ×100 (**B**) Invasive adenocarcinoma of case No.1 in table 2 was stained with control goat IgG (left panel) and anti-PVRL4 antibody (right panel) by paraffin tissue. Bar: 50 μm. Magnification: ×200. (**C**) Simple tubular adenocarcinoma of case No.2 in table 2 was stained with control IgG (left panel) or anti-PVRL4 antibody (light panel) by frozen tissue section. Bar: 50 μm. Magnification: ×200. (**D**) Primary tumor cells of case No.2 in table 2 were digested to single cells, and followed by inoculation with rMV-EGFP-SLAMblind at a MOI of 0.01. Photograph was taken at 2 dpi. Magnification: ×100. (**E**) Flow cytometry analysis of canine PVRL4 in primary CMT tissue. Tumor of case No.2 in table 2 were digested and stained with control IgG (gray histogram) or anti-PVRL4 antibody (white histogram). Main population was selected based on DAPI incorporation with FSC and SSC. Histogram indicates the expression level of PVRL4 in primary tumor cells. Around 46% of tumor cells were positive for PVRL4.

Figure 7. Expression of PVRL4 in normal tissues of whole body

(A) The mRNA level of canine PVRL4 were analyzed by real-time RT-PCR in normal 26 tissues. The data represent the mean ±SD from three independent experiments. This analysis was performed in triplicate. **(B-E)** 3 μm paraffin section of normal each tissue of was stained with anti-PVRL4 antibody as primary antibody. ImmPRESS Reagent anti goat IgG and DAB were used to visualize. Sections

were counterstained with Hematoxylin. Bar: 100 μm. Magnification: ×200. **(B)** Tongue; **(C)** Esophagus; **(D)** Bladder; **(E)** Renal pelvis**. (F, H, K, M)** Normal tissue section was stained with hematoxylin and eosin. Bar: 100 μm. Magnification: ×200 of (F) and ×100 of (H, K, M). **(F)** Lung and bronchus; **(H)** Epidermal tissue; **(K)** Fat pad; **(M)** Oral epithelium. **(G, I, J, L, N)** 3 μm paraffin section of normal skin of was stained with anti-PVRL4 antibody as primary antibody. PVRL4 was visualized using Alexa Fluor 488 rabbit anti-goat IgG (green) and nuclei were visualized with Hoechst33342 (blue). Magnification: ×720 of (G) and ×600 of (I, J, L, N). **(G)** Bronchial epithelium; **(I)** Epidermal cells; **(J)** Hair follicle cells; **(L)** Fatpad; **(N)** Oral epithelium.

Table

Table 1. A list of CMC cells used in this study

Name of cells	Pathological diagnosis	Source of cells	Expression of PVRL4
CF33	AC	Primary	+
AZACB	Complex AC	Primary	-
CBrC	Inflammatory AC	Primary	-
CHMp	Inflammatory AC	Primary	-
CHMm	Inflammatory AC	Pleural effusion	+
CTBp	Inflammatory AC	Primary	+
CTBm	Inflammatory AC	Metastatic RLN	+
CIPp	AC	Primary	-
CIPm	AC	Metastatic RLN	-

AC; Adenocarcinoma, RLN; Regional Lymph Node

Table 2. Expression of canine PVRL4 in clinical tissue samples

Case No.	Pathological diagnosis	Malignant or Benign	Expression of PVRL4
1	Invasive AC	Malignant	+
2	Simple Tubular AC	Malignant	+
3	Anaplastic Carcinoma	Malignant	−
4	Complex Adenoma	Benign	+
5	Complex Adenoma	Benign	+
6	Complex Adenoma	Benign	−
7	Complex Adenoma	Benign	−
8	Complex Adenoma	Benign	−
9	Adenoma	Benign	−
10	Adenoma	Benign	−
11	N.D.	N.D.	+

AC; Adenocarcinoma, N.D.; No Data

FIGURE

Figure 1-1

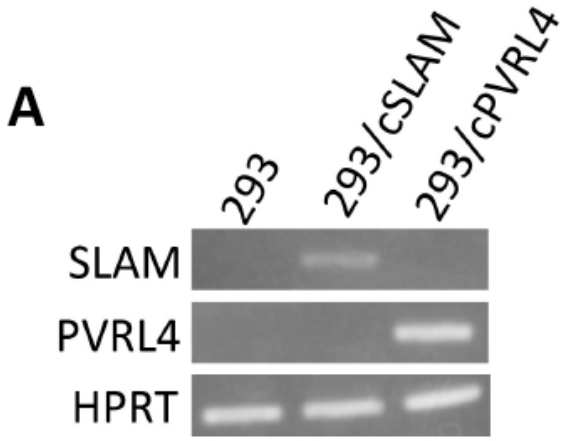
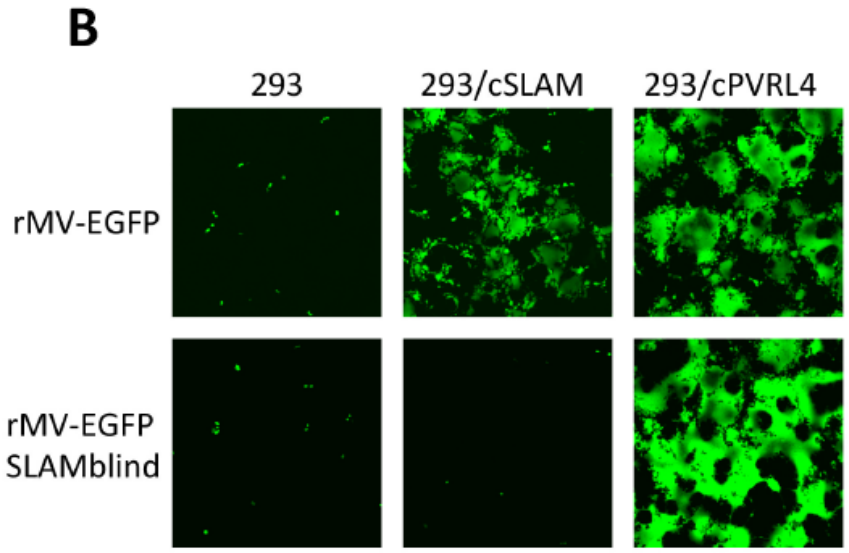


Figure 1-2


B
293
293/cSLAM
293/cPVRL4
rMV-EGFP
rMV-EGFP
SLAMblind

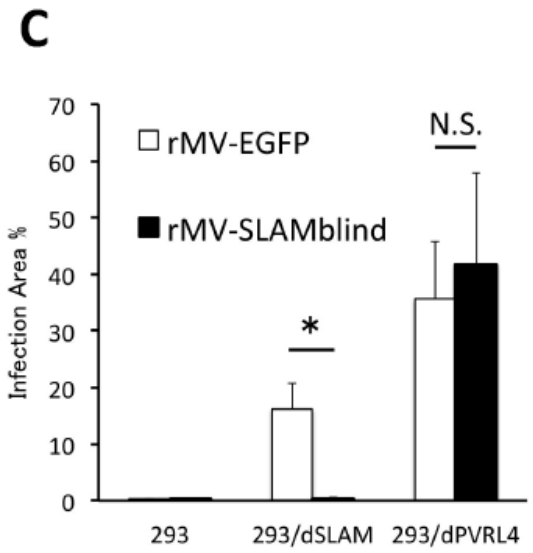
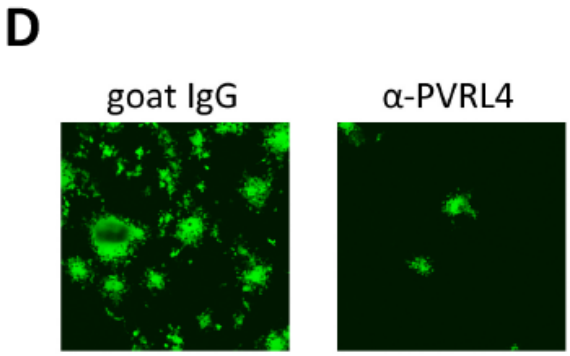

C
70
60
50
40
30
20
10
0
Infection Area %
rMV-EGFP
rMV-SLAMblind
N.S.
*
293
293/dSLAM
293/dPVRL4

Figure 1-3


D
goat IgG
α-PVRL4

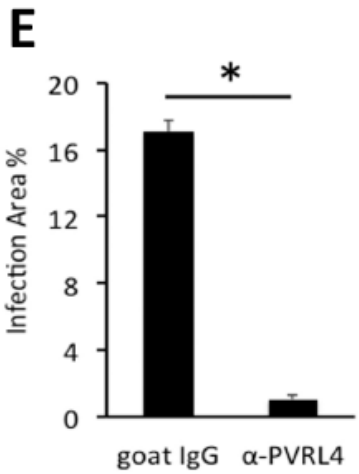

E
*
20
16
12
8
4
0
Infection Area %
goat IgG
α-PVRL4

Figure 2

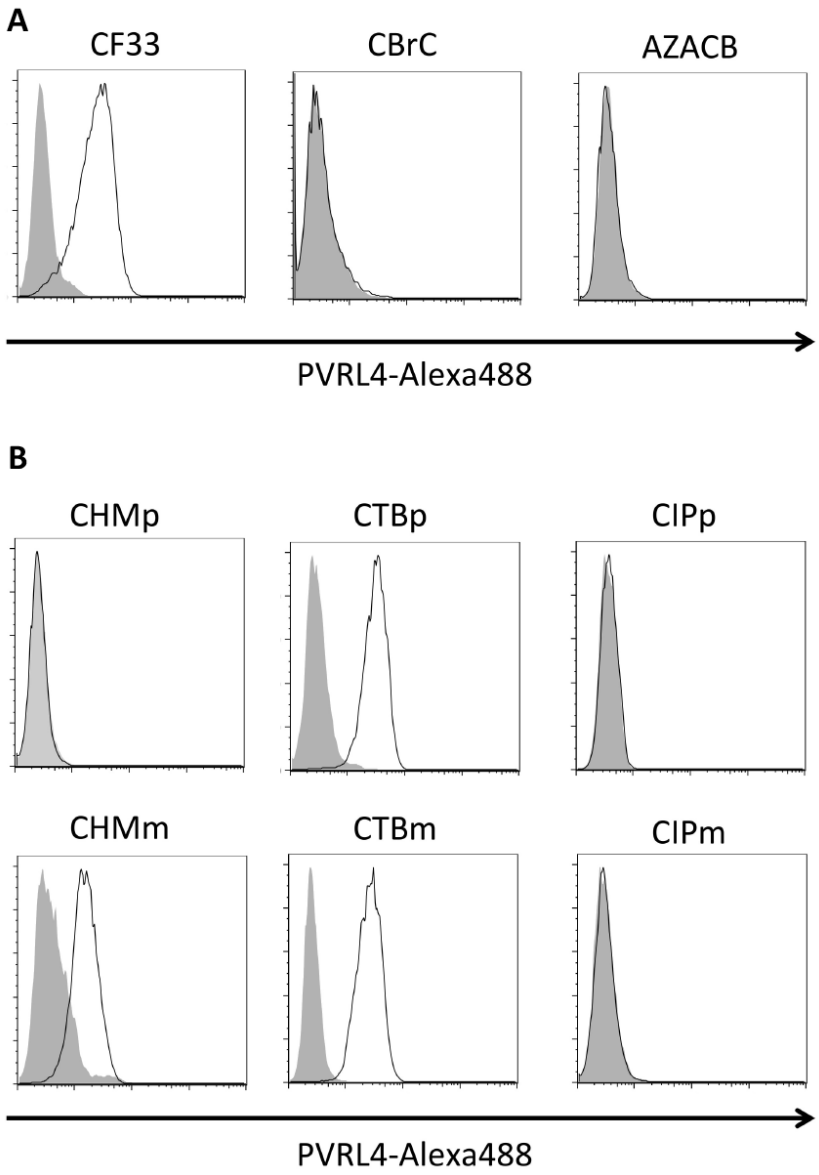

A
CF33
CBrC
AZACB
PVRL4-Alexa488
B
CHMp
CTBp
CIPp
CHMm
CTBm
CIPm
PVRL4-Alexa488

Figure 3-1

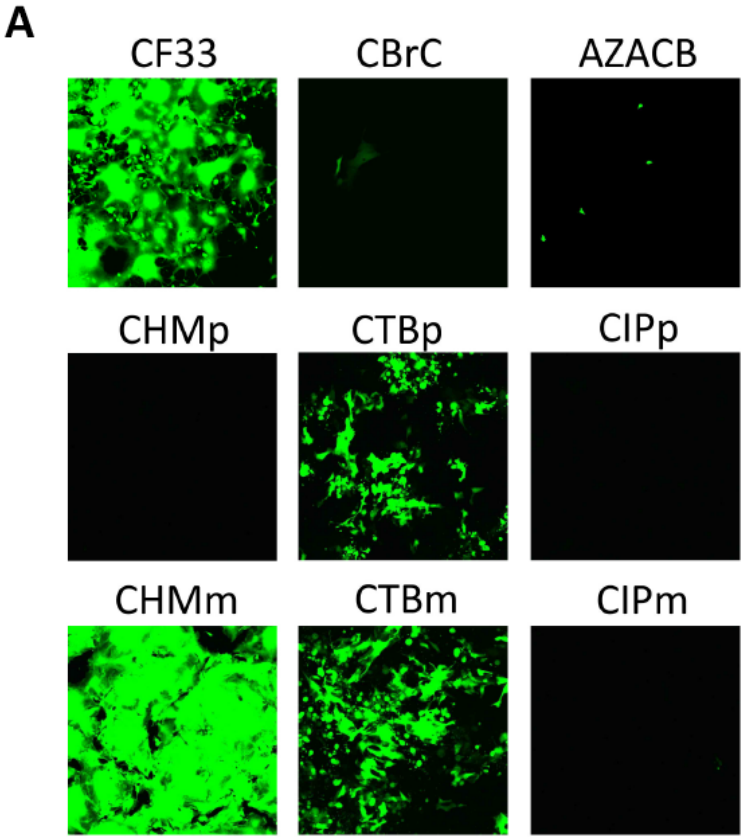


Figure 3-2

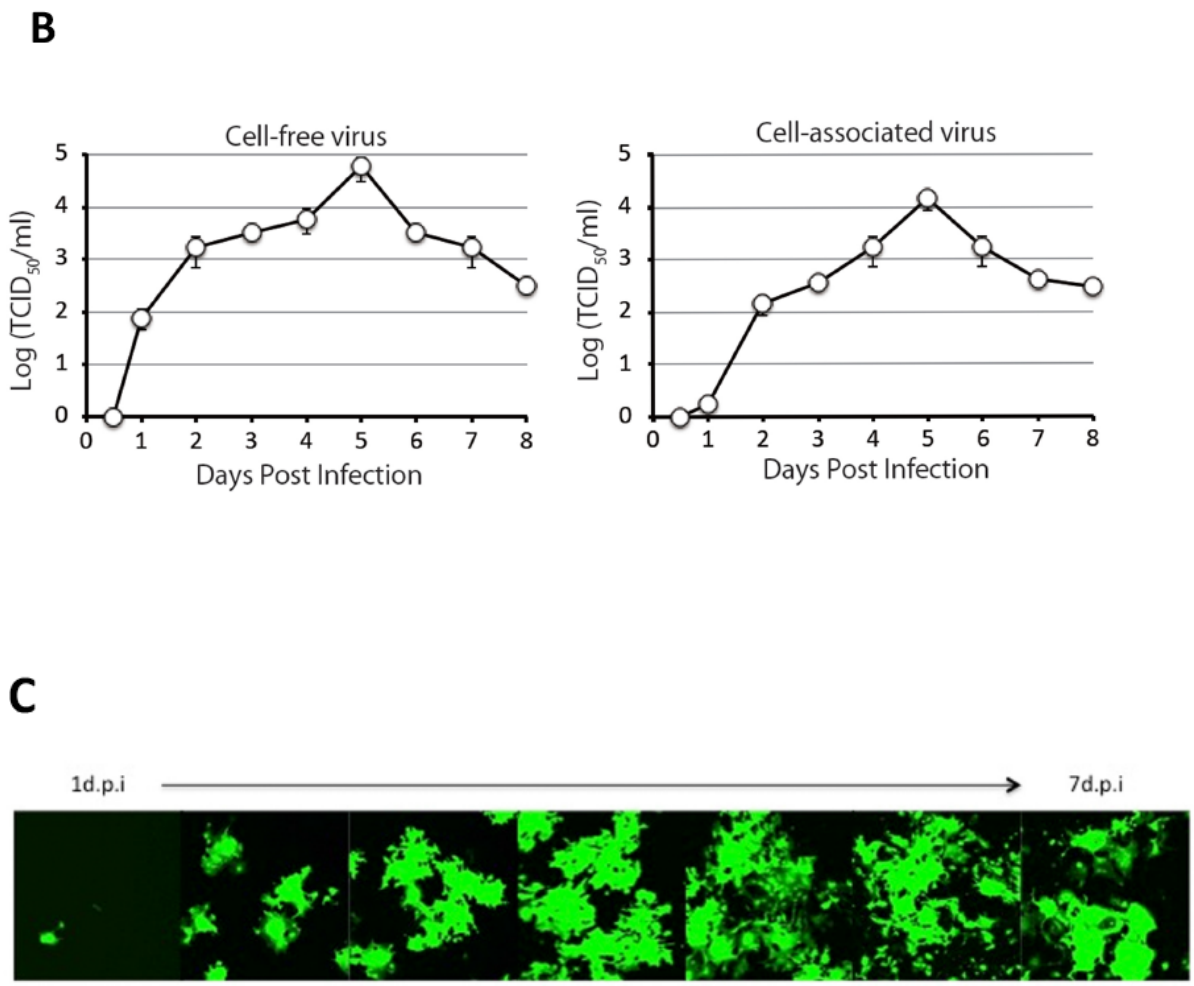

B
Cell-free virus
Log (TCID50/ml)
Days Post Infection
Cell-associated virus
Log (TCID50/ml)
Days Post Infection
C
1d.p.i
7d.p.i

Figure 4-1

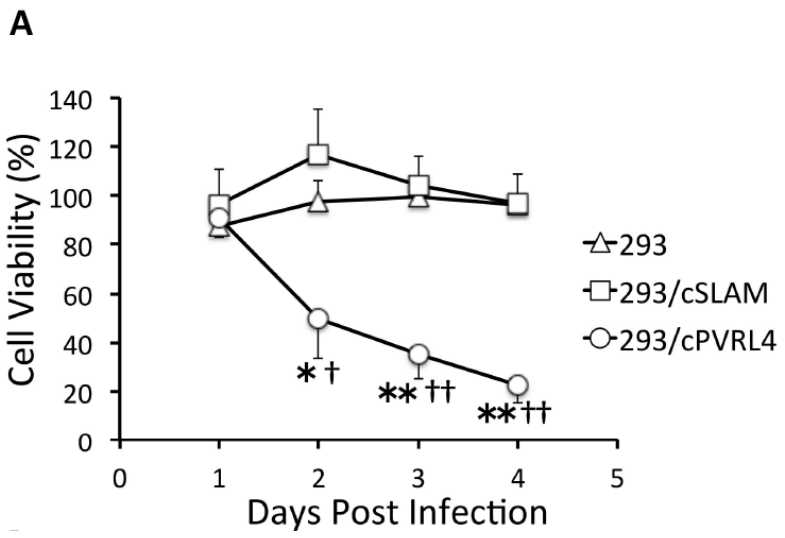

A
Cell Viability (%)
140
120
100
80
60
40
20
0
0
1
2
3
4
5
Days Post Infection
293
293/cSLAM
293/cPVRL4
*†
**††
**††

Figure 4-2

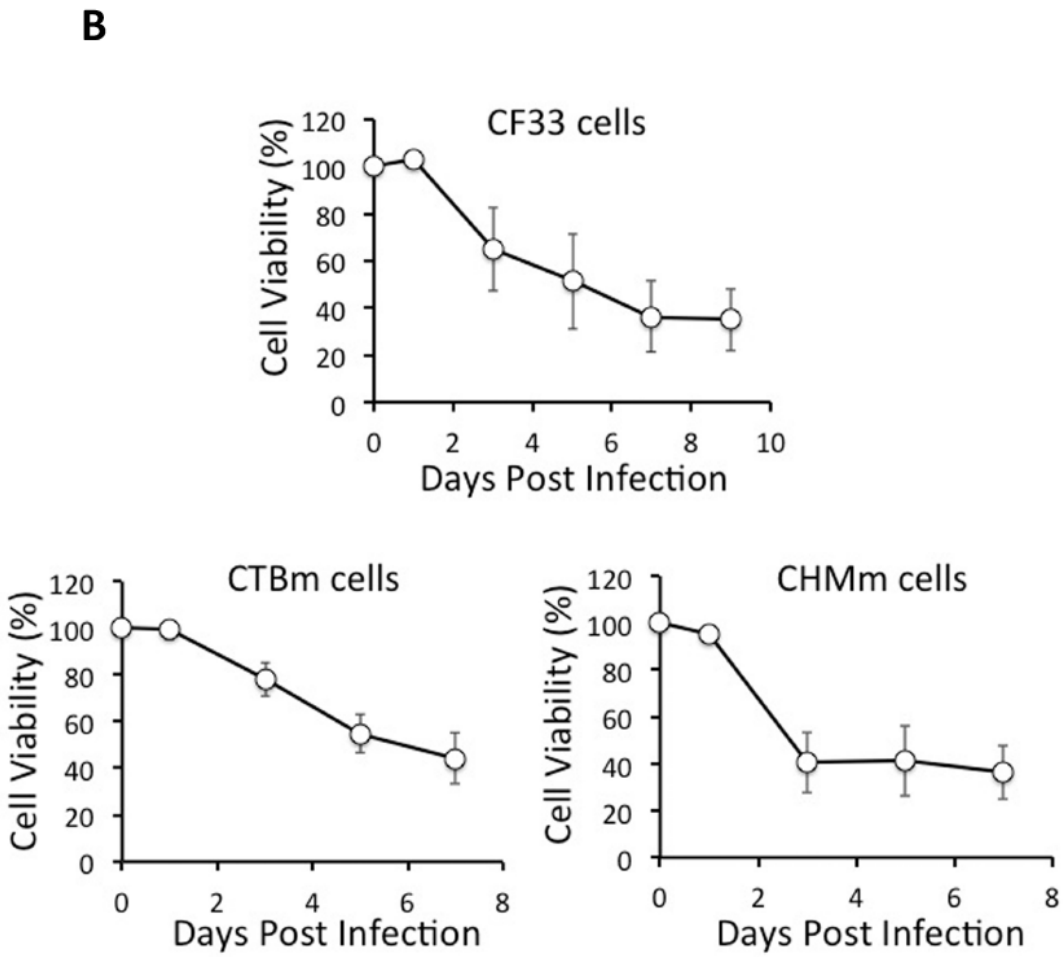

B
CF33 cells
Cell Viability (%)
120
100
80
60
40
20
0
0
2
4
6
8
10
Days Post Infection
CTBm cells
Cell Viability (%)
120
100
80
60
40
20
0
0
2
4
6
8
Days Post Infection
CHMm cells
Cell Viability (%)
120
100
80
60
40
20
0
0
2
4
6
8
Days Post Infection

Figure 5-1

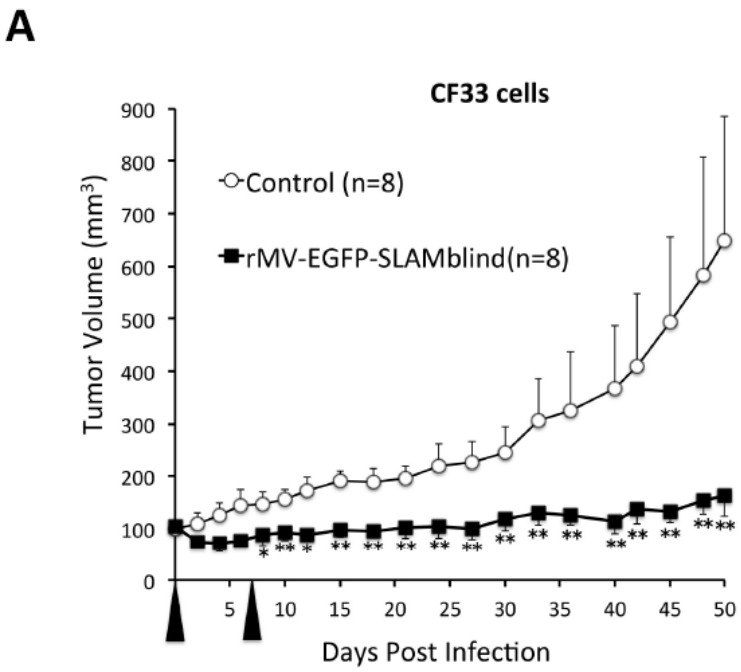

A
CF33 cells
Control (n=8)
rMV-EGFP-SLAMblind(n=8)
Tumor Volume (mm³)
900
800
700
600
500
400
300
200
100
0
5
10
15
20
25
30
35
40
45
50
Days Post Infection

Figure 5-2

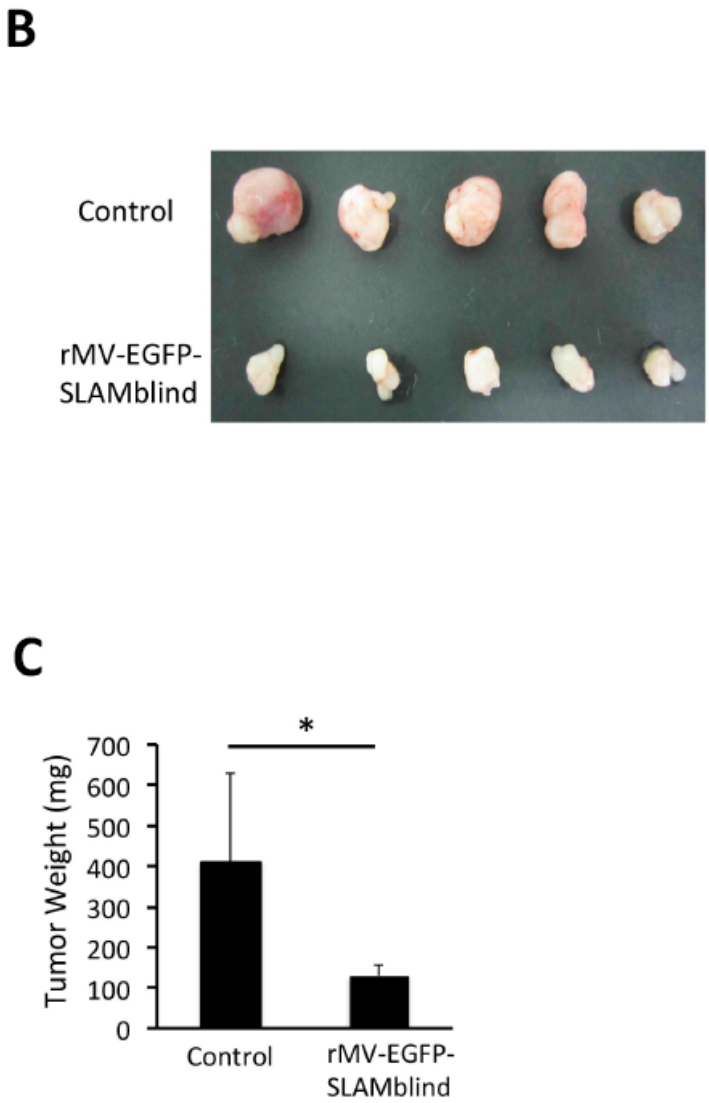

B
Control
rMV-EGFP-
SLAMblind
C
*
Tumor Weight (mg)
700
600
500
400
300
200
100
0
Control
rMV-EGFP-
SLAMblind

Figure 5-3

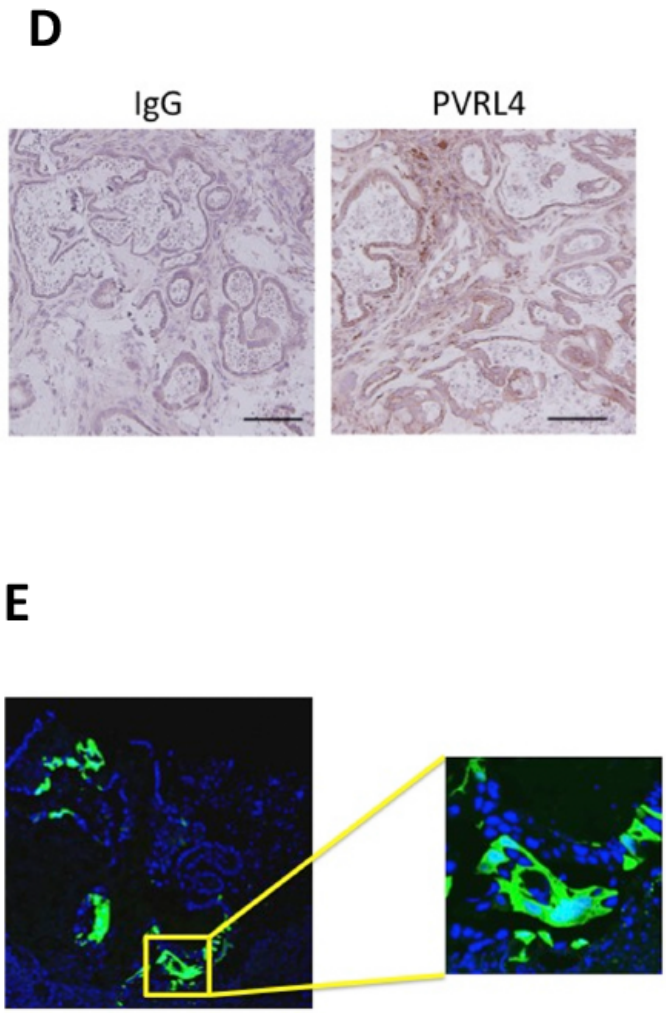


Figure 6-1

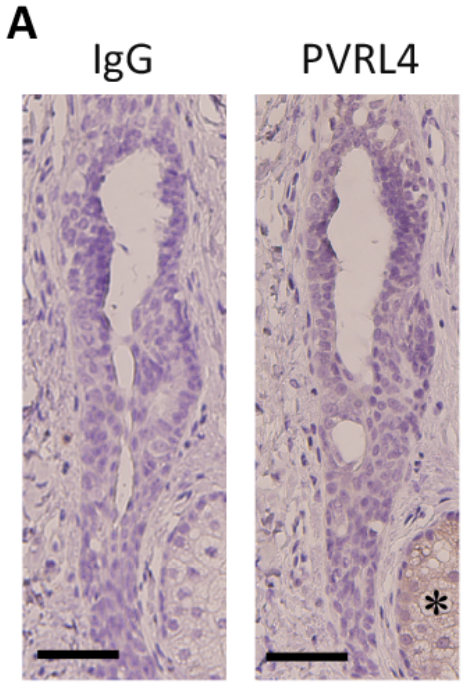


Figure 6-2

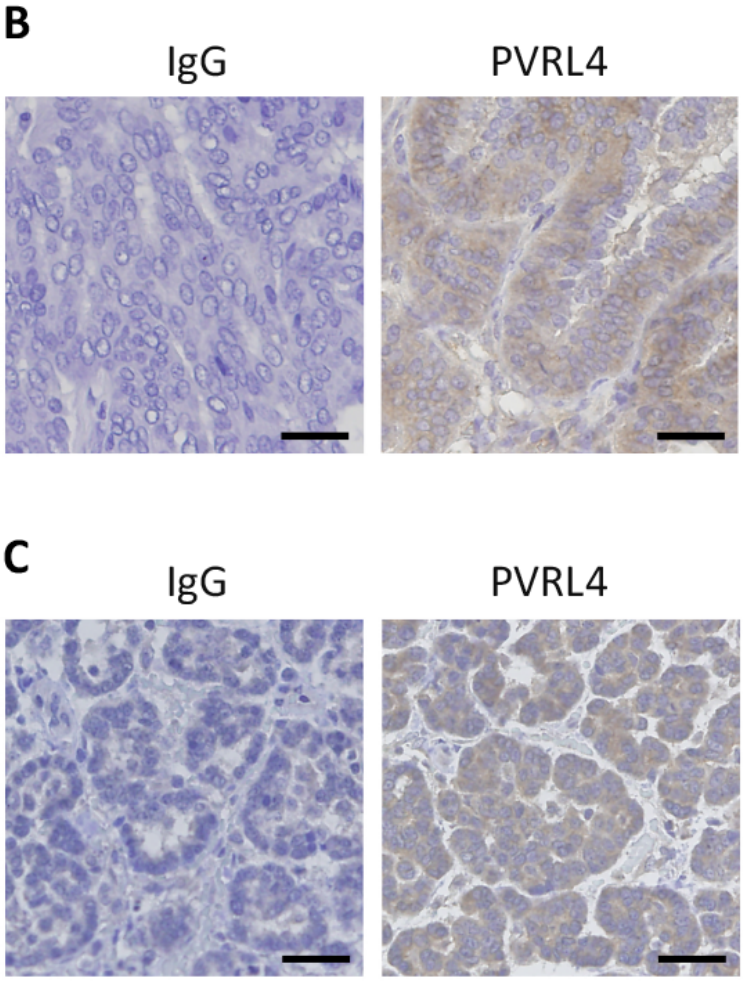


Figure 6-3

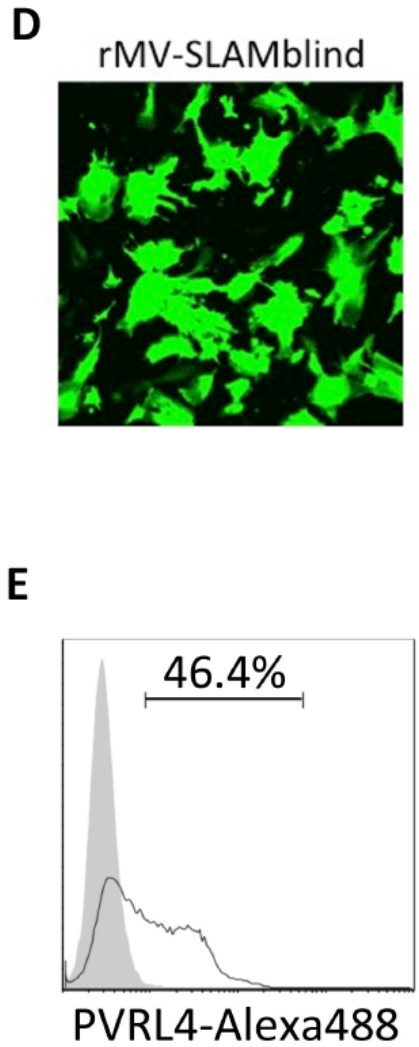


Figure 7-1

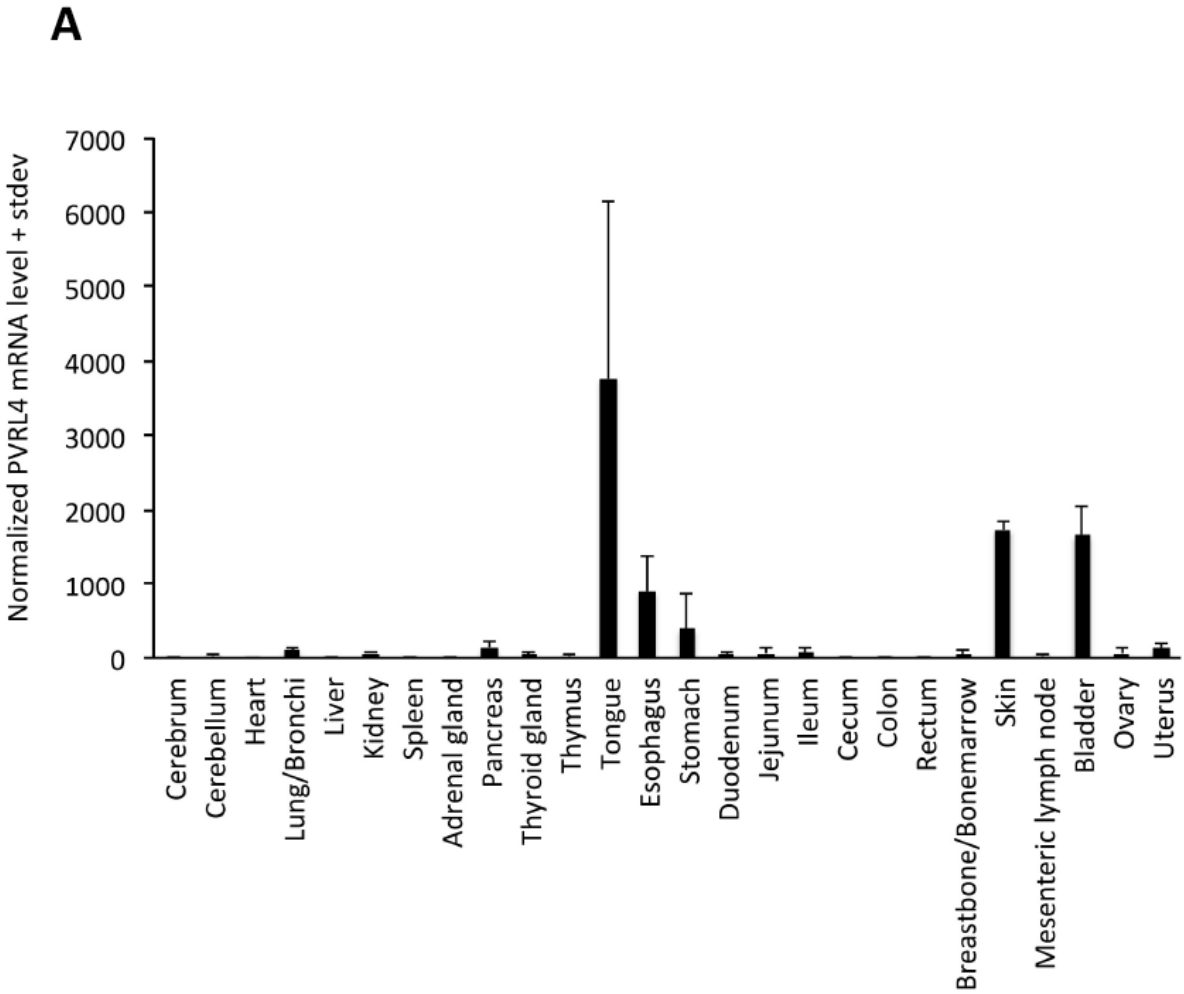

A
Normalized PVRL4 mRNA level + stdev
7000
6000
5000
4000
3000
2000
1000
0
Cerebrum
Cerebellum
Heart
Lung/Bronchi
Liver
Kidney
Spleen
Adrenal gland
Pancreas
Thyroid gland
Thymus
Tongue
Esophagus
Stomach
Duodenum
Jejunum
Ileum
Cecum
Colon
Rectum
Breastbone/Bonemarrow
Skin
Mesenteric lymph node
Bladder
Ovary
Uterus

Figure 7-2

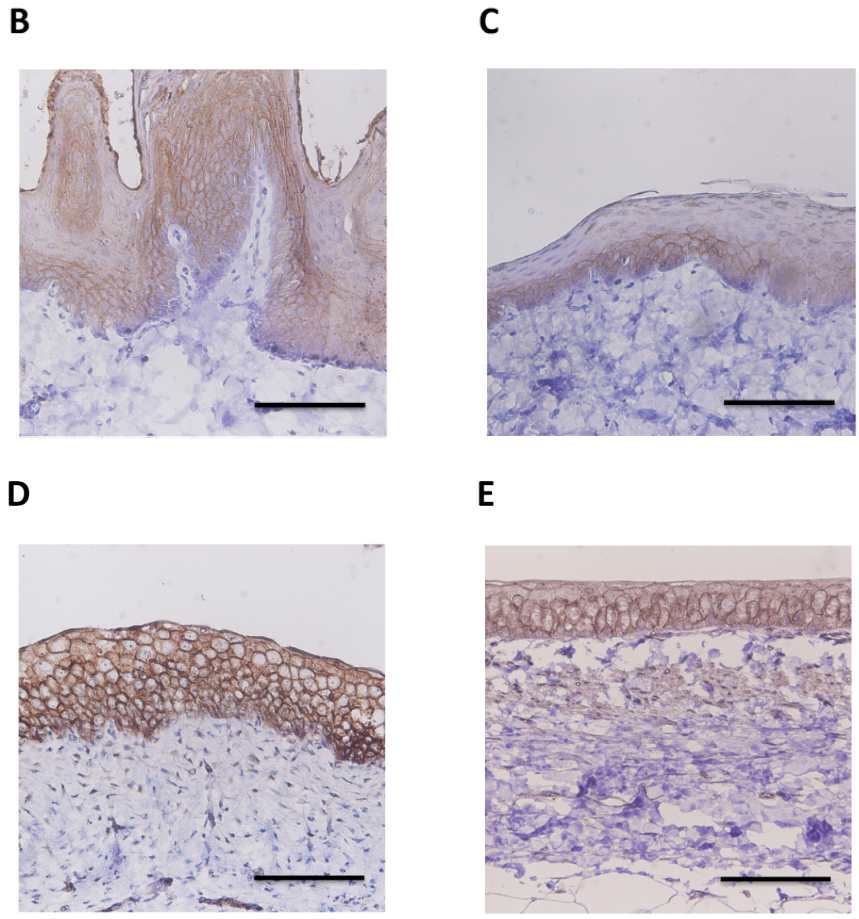


Figure 7-3

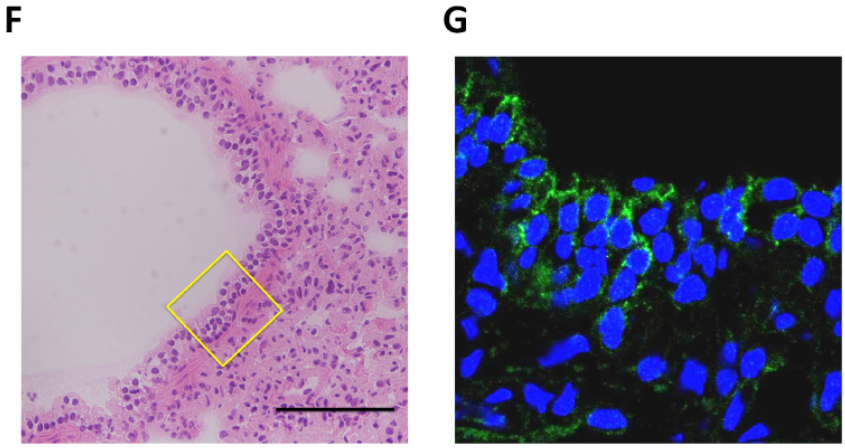


Figure 7-4

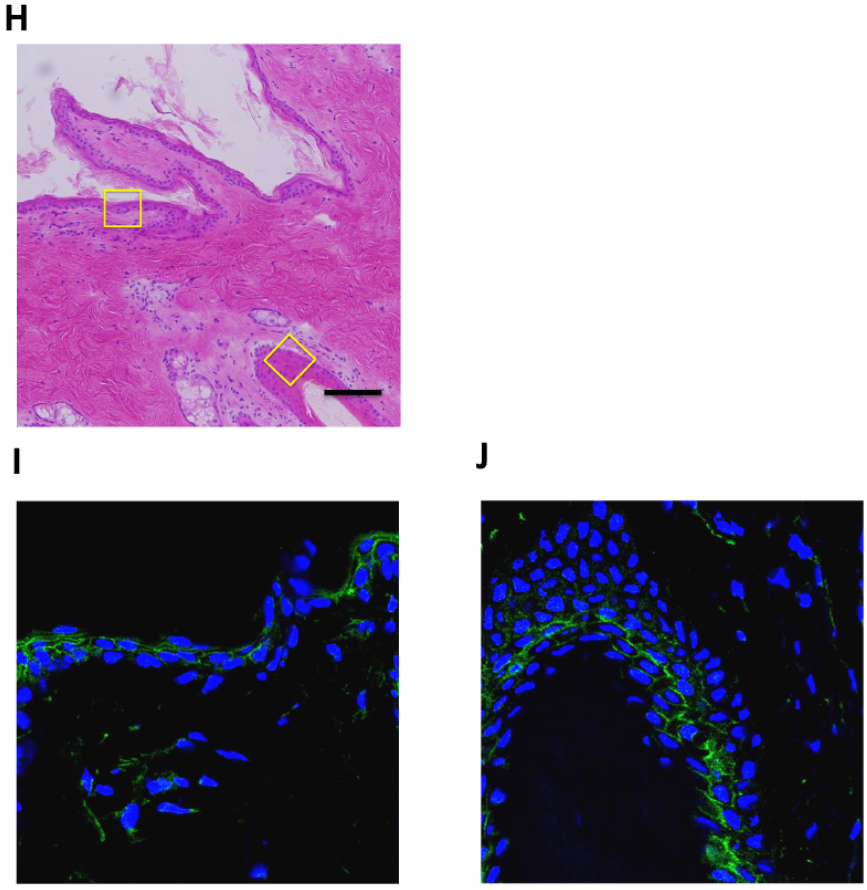
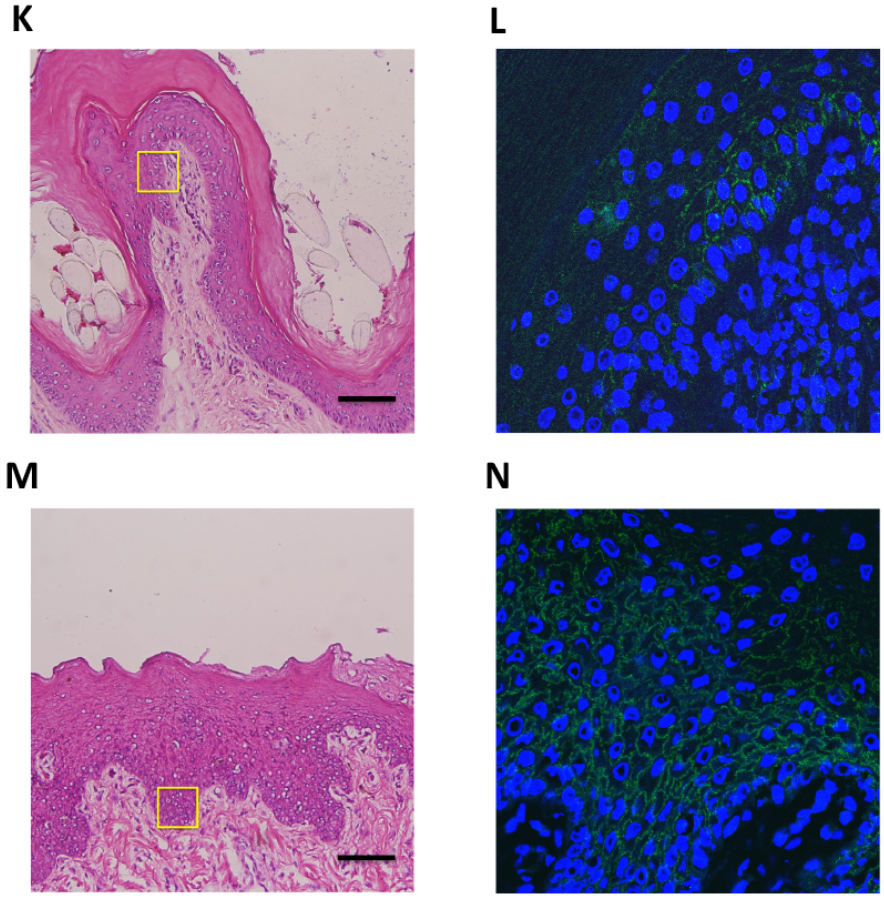


Figure 7-5



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ABSTRACT IN JAPANESE

論 文 の 内 容 の 要 旨

論文題目　Development of new therapy for canine mammary cancer with a recombinant measles virus

（イヌ乳がんに対する組換え麻疹ウイルスを用いた新規治療法の開発）

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イヌの乳腺腫瘍は雌犬で最も多く見られる腫瘍であり、その約半数は悪性の乳がんである。乳がんにおいては、手術を行った場合でも、その約半数は転移や再発により 1 年以内に死亡あるいは安楽死となる。特に、リンパ節あるいは肺への転移が認められた場合、その治療法は非常に限られ、多くの場合予後不良となる。このことから、イヌ乳がんに対する新たな治療法が必要であると考えられている。

近年、腫瘍溶解性ウイルスを用いたがんに対する新たな治療法が盛んに研究されており、ヒト医学領域では前臨床研究および臨床治験が積極的に行われている。一方で獣医学領域では、DNA ウイルスのアデノウイルス、ヘルペスウイルスおよびワクシニアウイルス、RNA ウイルスのレオウイルスおよびイヌジステンパーウイルス（CDV)において腫瘍溶解性ウイルスとしての有効性が報告されているが、これまで臨床現場で使用された例はごく限られている。CDV と共にパラミクソウイルス科・モルビリウイルス属に分類されている麻疹ウイルス(MV)は宿主ゲノムに組み込まれない事、細胞傷害性が強い事などからヒト医学領域において腫瘍溶解性ウイルスとしての有効性が期待されている。当研究室では、麻疹ウイルス野生株が、ヒト乳がん細胞に対して優れた抗腫瘍効果を持つ事を見出した。野生株 MV は、感染レセプターとして主に免疫系の細胞で発現している Signaling Lymphocyte Activation Molecule（SLAM)および一部の上皮系細胞やがん細胞で発現している Poliovirus receptor-related protein 4 (PVRL4)を利用する。MV の主な病原性は、ウイルスが SLAM を介して免疫細胞に感染することによる免疫抑制と、リンパ球を介して全身に伝播することにより発揮される。そこで当研究室では、SLAM に結合できなくなる変異を導入した組換えウイルス rMV-SLAMblind を作出し、このウイルスが生体内で免疫抑制を起こさず、さらに PVRL4 を介した乳がん細胞への感染・傷害性を保持する事を明らかにした。PVRL4 はイヌとヒト間で遺伝子の相同性が高く、MV の感染に重要なドメインは完全に保存されている。

そこで著者は、rMV-SLAMblind をイヌの乳がん治療に応用できるのではないかと考え、イヌ乳がんにおける PVRL4 の発現および rMV-SLAMblind のイヌ乳がんに対する抗腫瘍効果について検討を行った。

はじめに rMV-SLAMblind がイヌ PVRL4 を利用して細胞へ感染できるかを確認するために、HEK293 細胞にイヌ PVRL4 およびイヌ SLAM を発現させた安定発現細胞株を作製し、EGFP を組み込んだ rMV-EGFP-SLAMblind およびコントロールとして親株の rMV-EGFP を multiple of infection (MOI)=0.01 で感染させた。rMV-EGFP がイヌ SLAM 発現細胞に感染したのに対し、rMV-EGFP-SLAMblind ではその感染性は認められなかった。一方で、イヌ PVRL4 発現細胞に対する感染性についてはどちらのウイルスでも維持されていた。さらに抗 PVRL4 抗体でレセプターをブロックする事により感染が阻害されるかを観察したところ、コントロール IgG を加えた時と比較し、rMV-EGFP-SLAMblind の感染が明らかに阻害された。以上の事から、rMV-EGFP-SLAMblind はレセプターとしてイヌ SLAM ではなくイヌ PVRL4 を介して感染する事が示された。

イヌ乳がん細胞において PVRL4 が発現しているかを調べるために、イヌ乳がん細胞株 9 株を用いて、フローサイトメトリーで解析した。その結果、CF33 細胞、CHMm 細胞、CTBp 細胞および CTBm 細胞の 4 株で発現が認められた。CHMp 細胞と CHMm 細胞、CTBp 細胞と CTBm 細胞および CIPp 細胞と CIPm 細胞はそれぞれ同じ患畜の原発巣あるいは転移巣より樹立された細胞株である。CHMm 細胞および CTBm 細胞においても発現が認められた事から、乳がんの原発巣だけではなく転移巣においてもイヌ PVRL4 が発現している事が示された。また、これらの 9 細胞株に対して、rMV-EGFP-SLAMblind を MOI = 2 で播種し、EGFP を指標として感染の有無を観察した。その結果、PVRL4 が発現している細胞に対してのみ効率良く感染した。イヌ乳がん細胞でのウイルスの増殖を評価するために、rMV-EGFP-SLAMblind の感染効率の高かった CF33 細胞に、MOI = 0.01 で感染させ、経時的に回収したウイルスの力価を測定する事により増殖曲線を作成した。培養上清中ウイルスおよび細胞中ウイルス共に感染 5 日目で最高のウイルス力価を示し、イヌ乳がん細胞において、rMV-EGFP-SLAMblind がよく増殖できることが確認された。

rMV-EGFP-SLAMblind の効率のよい感染が認められた 3 種のイヌ乳がん細胞株におけるウイルスの細胞傷害性を WST-1 assay により評価した。その結果、感染後 7 日で、CF33 細

胞の 71%、CTBm 細胞の 56%および CHMm 細胞の 68%が傷害された。この結果から、*in vitro* において、rMV-EGFP-SLAMblind は PVRL4 発現イヌ乳がん細胞に対して強い細胞傷害性を有する事が示された。

In vivo での rMV-EGFP-SLAMblind の抗腫瘍効果を評価するために、SCID (severe combined immune deficient)マウスの皮下に CF33 細胞を移植した xenograftmodel を作製し、治療実験を行った。細胞接種後、腫瘍が約 100mm^3 に達したところで、10^6 $TCID_{50}$ の rMV-EGFP-SLAMblind を腫瘍内に接種した。7 日後にウイルスを再度投与し、経時的に腫瘍の体積を測定した。その結果、rMV-EGFP-SLAMblind 投与群では投与していない群に比べて顕著に腫瘍の増殖が抑制されていた。すべてのマウスを、ウイルス接種後 50 日で安楽殺し、腫瘍を摘出しそれらのサイズを比較したところ、明らかにウイルス投与群では小さくなっており、重量についても有意な差が認められた。また、腫瘍内細胞におけるウイルス感染の有無を確認するために、一部のマウスをウイルス投与 4 日後に安楽殺し、EGFP を指標として観察したところ、腫瘍細胞において感染が認められ、MV 感染による典型的な cytopathic effect である多核巨細胞の形成も認められた。これらの結果から rMV-EGFP-SLAMblind がイヌ乳がんに対して、*in vivo* においても抗腫瘍効果を示す事が示された。

臨床検体での PVRL4 の発現を調べるために、手術により摘出された 11 検体の乳腺腫瘍組織およびイヌ正常乳腺組織の凍結切片あるいはパラフィン切片を作製し、免疫組織化学染色(IHC)を行った。その結果、正常組織においては乳腺上皮細胞および筋上皮細胞共にイヌ PVRL4 の発現は認められなかった。一方で、イヌ乳腺腫瘍組織では悪性腫瘍の 67%、良性腫瘍の 29%、全体で 45%の腫瘍で PVRL4 の発現が認められた。また、PVRL4 陽性の腫瘍組織から初代培養細胞を作製し、rMV-EGFP-SLAMblind の感染性を評価した。ウイルスを MOI=0.01 で感染させ、EGFP の蛍光を観察したところ、効率よく感染しているのが確認された。以上のことから、臨床検体において半数近い症例で PVRL4 が発現しており、発現の認められた細胞に対しては効率良く rMV-EGFP-SLAMblind が感染することが示された。

ウイルスのトロピズムと正常組織でのレセプター発現組織は密接に関係していることから、rMV-SLAMblind 療法の副作用を予測する為に、正常イヌ組織での PVRL4 の発現について、mRNA の発現および免疫染色により解析を行った。その結果、mRNA については舌・食道・胃・皮膚および膀胱において高発現が認められ、肺・腎・膵および子宮において低発現では

あるが認められた。mRNA が検出された組織についてタンパク質の発現を観察するために、免疫染色を行ったところ、重層扁平上皮により構成される舌・食道では、基底層および有棘層に局在して発現が認められた。一方で、移行上皮から構成される膀胱および腎盂では、上皮細胞全体に認められた。皮膚においては、表皮細胞において発現認められた他、毛包細胞において強いシグナルが認められた。肺では、気管支の上皮細胞においてのみ局在しているのが確認された。これまで、イヌにおいては、脳・肺・腸管・胃・腎および膀胱での発現が報告されていた。しかしながら、本研究では脳および腸管での発現は確認できなかった一方で、新たに舌・皮膚・食道で発現を認めたほか、PVRL4 の上皮細胞での局在についても明らかにした。

本研究では、組換え MV を用いたウイルス療法がイヌ乳がんに対する治療法となりうるかを検討した。その結果、rMV-EGFP-SLAMblind はイヌ PVRL4 発現細胞に対して、効率良く感染し、*in vitro* および xenograft モデルにおいて、顕著な抗腫瘍効果を示した。獣医学領域において、乳がんに対する治療法は外科的処置が第一選択ではあるが、転移により完全に除去できない場合には、副作用の強い抗がん剤による治療が中心となり、ヒトで有効性が確認されている分子標的薬については十分なエビデンスが報告されていない。興味深い事に、本研究では転移巣から樹立された細胞株である CHMm 細胞および CTBm 細胞にイヌ PVRL4 が発現しており、rMV-EGFP-SLAMblind がこれらの細胞株に傷害性を示した。特に CHMm 細胞については、同じ患畜の原発巣から樹立された CHMp 細胞ではイヌ PVRL4 の発現が認められなかったことから、イヌ PVRL4 が転移性乳がん、すなわちより悪性の乳がんに発現する可能性を示唆している。この事は、イヌ乳腺腫瘍組織の IHC においてイヌ PVRL4 が悪性乳がんにおいて発現頻度が高い傾向が認められた結果からも支持される。

PVRL4 は正常組織において、一部の上皮細胞において発現が認められた。しかしながら、我々のグループによって行った正常イヌに対する rMV-SLAMblind の安全性試験では、PVRL4 発現部位および血中、尿中および糞便中にウイルスの排出が認められない事を確認している。このことから、rMV-SLAMblind 療法はイヌに対して安全であると考えられた。

以上より、rMV-SLAMblind 療法は、従来の治療法では治癒困難な腫瘍に対する、有望な新規治療法になると期待される。