



## NOTE

Clinical Pathology

# Molecular cloning of canine Wilms' tumor 1 for immunohistochemical analysis in canine tissues

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**ABSTRACT.** Wilms' tumor 1 (WT1) expression has been investigated in various human cancers as a target molecule for cancer immunotherapy. However, few studies have focused on WT1 expression in dogs. Firstly, cDNA of canine WT1 (cWT1) was molecularly cloned from normal canine kidney. The cross-reactivity of the anti-human WT1 monoclonal antibody (6F-H2) with cWT1 was confirmed via Western blotting using cells overexpressing cWT1. Immunohistochemical staining revealed that cWT1 expression was detected in all canine lymphoma tissues and in some normal canine tissues, including the kidney and lymph node. cWT1 is a potential immunotherapy target against canine cancers.

**KEY WORDS:** cancer, canine, immunohistochemical staining, Wilms' tumor 1

Cancer is the most common cause of death in dogs, and even though a lot of efforts have been put into the discovery of novel canine cancer therapeutics, only a hand full of these treatments are available in actual clinical practice. During last two decades, cancer immunotherapy has been expected to be new kinds of therapy, where various therapeutic approaches utilizing the immune system to fight against cancer have been attempted. Among these approaches, peptide immunotherapy is expected to have the highest potential. Along with the development of peptide immunotherapy, a large number of tumor-associated antigens (TAAs) have been identified in human cancer. The Wilms' tumor 1 (WT1) protein is one of the most common TAAs found in human cancers [3].

The WT1 gene was first identified in 1990 and categorized as a tumor-suppressor gene, because WT1 gene deletions and mutations were found in Wilms' tumor, a common renal tumor in children. WT1 plays an important role in normal cellular development, differentiation and cell survival [2, 5, 11]. High levels of wild-type WT1 expression were detected in various kinds of human cancers including hematopoietic malignancies, such as leukemia and myelodysplastic syndromes, and solid cancers, such as brain cancer, neuroblastoma, lung cancer, breast cancer, head and neck squamous cell carcinoma, thyroid cancer, esophageal cancer, ovarian carcinoma and renal cell carcinoma [14, 20]. Several studies have shown that induction of WT1-specific cytotoxic T lymphocytes (CTL) by the WT1 peptide vaccine is clinically effective in the treatment of human cancer. It is reported that WT1 peptide vaccination in combination with imatinib decreased bcr-abl transcript level in chronic myeloid leukemia patient and this combination did not induce serious adverse effects [12, 13]. Oka *et al.* reported the outcome of a phase I clinical study of WT1 peptide immunotherapy in patients with breast cancer, lung cancer, myelodysplastic syndrome and acute myeloid leukemia [15]. Clinical responses, such as reduction in the number of tumor cells and tumor sizes, were achieved in twelve out of twenty patients. The study also confirmed a clear correlation between the increment of WT1-specific CTL and clinical responses.

On the other hand, there have been few reports on the expression of canine WT1 (cWT1) in dogs [7, 8, 17], and none of those reports was related to canine tumors. Therefore, evaluation of cWT1 expression in canine tumors will be helpful in the development of immunotherapy for dogs. In this study, we molecularly cloned the cWT1 gene and examined its cross-reactivity

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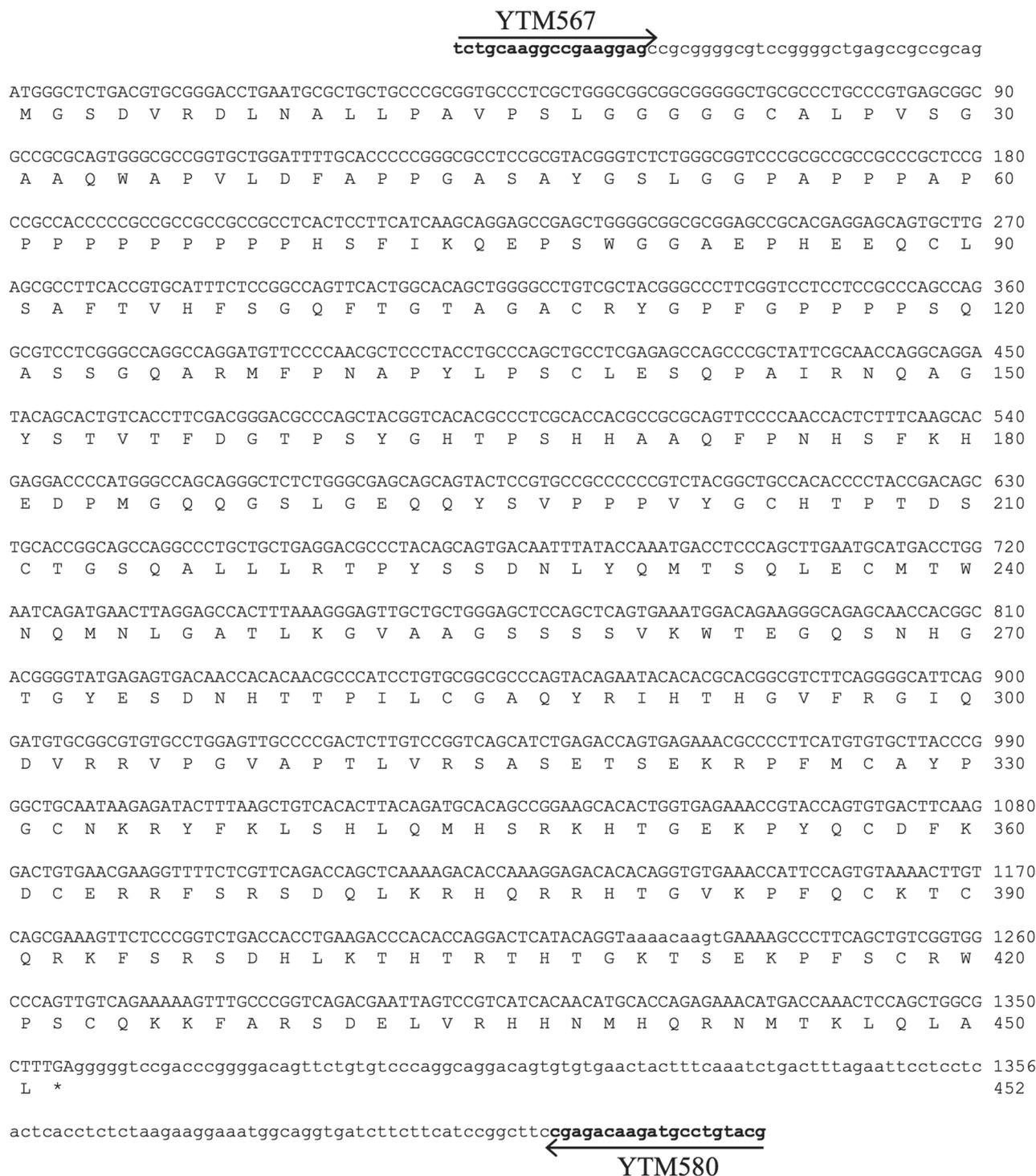
with an anti-human WT1 antibody. cWT1 expression was also assessed in canine lymphoma tissues, as well as in normal canine tissues.

Firstly, the cDNA of cWT1 was molecularly cloned for sequence analysis and protein overexpression. One microgram of total RNA isolated from normal canine kidney tissue was treated with Turbo DNA-free (Ambion Life Technologies, Austin, TX, U.S.A.) and transcribed into cDNA using Superscript III (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) according to manufacturers' instructions. Oligo dT primers were used to prime the first-strand synthesis for each reaction. Primers for the cDNA amplification of cWT1, YTM567 (5' TCTGCAAGGCCGAAGGAG 3') and YTM580 (5' CGTACAGGCATCTGTCTCG 3'), were designed based on predicted sequences of cWT1 in the canine genomic database (GenBank Accession No. NW\_876266.1). Using this primer pair, the cWT1 gene was amplified from the normal canine kidney cDNA using KOD plus kit (Toyobo, Osaka, Japan) according to manufacturer's instructions. Predenaturation at 94°C for 2 min was followed by 35 cycles of PCR amplification, which consists of denaturation at 98°C for 10 sec, annealing at 56°C for 30 sec, extension at 68°C for 2 min and a final extension at 68°C for 10 min. PCR generated a single DNA fragment with an expected size of approximately 1,500 bp. The gel-purified PCR product was inserted into SmaI restriction sites of pBluescript SK (-) vector (pBS-cWT1). The constructed vector was sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Perkin-Elmer, Foster City, CA, U.S.A.) and analyzed using ABI377 automated DNA sequencer at Yamaguchi University Center for Gene Research. Nucleotide sequence analysis of the full-length cWT1 revealed a cDNA clone of 1,559 bp that has an open reading frame of 1,356 bp, encoding 451 amino acids (Fig. 1). The nucleotide sequence of cWT1 was 94 and 91% identical to the human and mouse WT1. When compared with the predicted partial nucleotide sequence of cWT1 (GenBank Accession No. NW\_876266.1), 11 mismatched nucleotides were found in the nucleotide sequence of the coding region of cWT1. The alignment of the predicted amino acid sequence of the cWT1 cDNA, the human WT1 cDNA and the mouse WT1 cDNA are shown in Fig. 2. The deduced amino acid sequence of the cWT1 cDNA was highly homologous (99 and 97%) with that of the human and mouse WT1 polypeptides.

In order to evaluate the cross-reactivity of anti-human WT1 monoclonal antibody (6F-H2) with cWT1, cWT1 was transiently overexpressed in human embryonic kidney (HEK) 293T cells. The expression vector was constructed by inserting BamHI and EcoRV-digested segments of the pBS-cWT1 into a mammalian expression vector pcDNA3.1 (+) (pcDNA3.1 (+)-cWT1). Empty vector and pcDNA3.1 (+)-cWT1 were transfected into HEK293T cells using Trans-it LTI (TAKARA BIO Inc., Otsu, Japan) according to manufacturer's instructions. The cells were collected 48 hr after transfection, lysed in lysis buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP40 and protease inhibitor (Complete, Mini, EDTA-free: Roche Diagnostics K.K., Tokyo, Japan)] at 4°C for 15 min and centrifuged at 20,000 g at 4°C for 15 min before supernatant (cell lysate) was collected. Protein concentration was measured using Micro BCA™ Protein Assay Reagent Kit (Thermo Scientific, Yokohama, Japan) according to the manufacturer's instructions. Samples of equal concentration were subjected to SDS-PAGE (9% acrylamide gel) and subsequent membrane blotting (Hybond™ ECL membrane, GE Healthcare Japan, Tokyo, Japan). Membrane was blocked with blocking buffer [Tris-buffered saline with 0.05% Tween 20 (TBS-T) and 5% skimmed milk] at RT for 1 hr before incubation with a primary antibody; mouse monoclonal anti-human WT1 antibody (6F-H2, Dako, Glostrup, Denmark, 1:500 dilution in 0.5% skimmed milk/TBS-T) at 4°C overnight or goat anti-actin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A., 1:2,000 dilution in 0.5% skimmed milk/TBS-T) at RT for 2 hr. Washing with TBS-T was carried out for 10 min three times before incubation with horseradish peroxidase-conjugated secondary antibodies; goat anti-mouse IgG antibody (Zymed Laboratories Inc., South San Francisco, CA, U.S.A., 1:4,000 dilution in 0.5% skimmed milk/TBS-T) or rabbit anti-goat IgG antibody (Bethyl Laboratories Inc., Montgomery, TX, U.S.A., 1:4,000 dilution in 0.5% skimmed milk/TBS-T) at RT for 1 hr. Washing with TBS-T was repeated again three times, and membranes were immersed in Western Lightning Chemiluminescence reagent Plus (Perkin-Elmer) for band visualization. The anti-human WT1 antibody, 6F-H2, recognized a band of the expected molecular weight at 50–55 kDa in the HEK293T cells transfected with pcDNA3.1 (+)-cWT1, but not in the control using pcDNA3.1 (+) (Fig. 3). This result strongly indicated that 6F-H2 cross-reacted with cWT1.

cWT1 expression was evaluated using 26 normal tissue specimens of various organs from 15 healthy dogs and 22 tumor specimens from 22 dogs diagnosed with lymphoma. The specimens, which were obtained from the laboratories of veterinary pathology of Yamaguchi University and Gifu University, were fixed in formalin and embedded in paraffin before immunohistochemical staining (IHC) was performed. IHC was carried out following the method described in a previous report [16]. Anti-WT1 antibody (6F-H2) or normal mouse IgG1 (Santa Cruz Biotechnology, Inc., Dallas, TX, U.S.A.) was used as the primary antibody, followed by treatment with horseradish peroxidase (HRP)-conjugated secondary antibody, goat anti-mouse IgG antibody (Histofine Simple Stain MAX PO: Nichirei Corporation, Tokyo, Japan). Finally, color was developed using freshly prepared 3,3-diaminobenzidine tetrachloride (Roche Diagnostics K.K., Tokyo, Japan). The slides were counterstained for 1 min with Carazzi's hematoxylin, washed in distilled water, dehydrated in graded baths of ethanol, cleared in xylene and mounted with xylene-based mounting solution (Matsunami, Osaka, Japan).

IHC results of the normal tissue specimens are summarized in Table 1. cWT1 was clearly detected in the glomerular cells, especially in the outer zone, of the kidney (Fig. 4A). According to the localization site, WT1-positive cells are considered to be podocytes, as reported [19]. Furthermore, WT1-positive signals were weakly detected in renal tubules, as observed in normal rat kidneys [6]. It is still unclear whether this weak signal is significant or not, and a future study will shed light on this notion. In addition, testis and lymph node (Table 1) were stained positively for cWT1. On the other hand, cWT1 expression was not detected in the heart, spleen, prostate, skeletal muscle and skin. All of the 22 canine lymphoma tissue specimens, irrespective of the immunophenotypes, anatomic sites and historical grade, were positively stained for cWT1 (Fig. 4C and 4D, Table 2). Lymphocytes in all the tumor specimens, as well as the normal control lymph nodes of healthy dogs, were stained in a similar pattern (Fig. 4B).



**Fig. 1.** Nucleotide sequence (top line) and the predicted amino acid sequence (bottom line) of cWT1. Letters in upper case represent the coding region of cWT1, and letters in lower case represent the non-coding region. Asterisk represents the stop codon. Numbers on the right refer to the nucleotide position of the cDNA of cWT1 (top line) and the amino acid sequence position (bottom line). Arrows indicate the primers involved.

In this study, we showed that the mouse anti-human WT1 monoclonal antibody (6F-H2) cross-reacted with cWT1. This was consistent with the description in the product specification, where the antigen binding site of 6F-H2 is located at the N-terminal amino acids 1–181 of the human WT1 and is expected to bind to cWT1. A previous report has used a polyclonal antibody against human WT1 for IHC in canine tissue specimens, but the specificity of the antibody to cWT1 was never shown [7]. Western blotting of canine cell lines carried out in our lab using an anti-human WT1 polyclonal antibody (C-19) has revealed multiple non-specific bands (data not shown). The specificity of the 6F-H2 antibody to cWT1 that was shown in this study will become useful

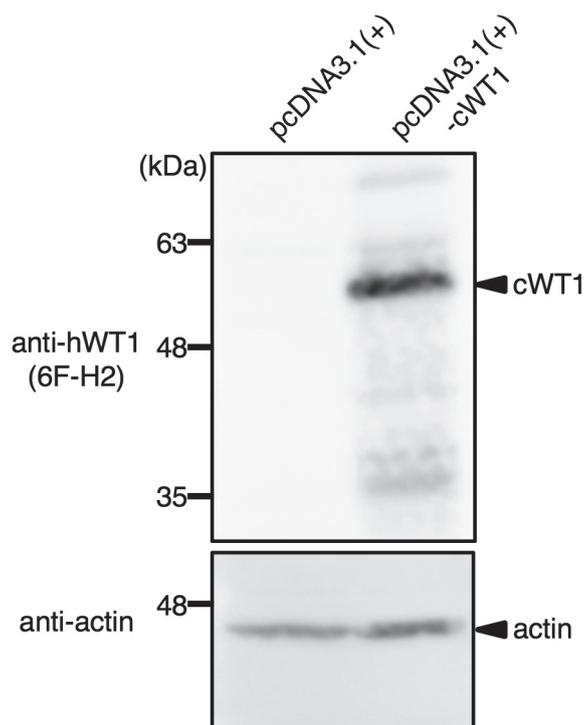
Dog	MGSDVRLNALLPAVPSLGGGGG-CALPVSGAAQWAPVLDFAAPPASAYGSLGGPAPPPA	59
Human	MGSDVRLNALLPAVPSLGGGGG-CALPVSGAAQWAPVLDFAAPPASAYGSLGGPAPPPA	59
Mouse	MGSDVRLNALLPAVSSLGGGGGGCGLPVSGARQWAPVLDFAAPPASAYGSLGGPAPPPA *****.******.*****.*****.*****.*****.*****.*****.*****.*****.*****	60
Dog	PPPPPPPPPHSFIKQEPSWGGAEPEHEEQCLSAFTVHFSGQFTGTAGACRYGPFPPPPS	119
Human	PPPPPPPPH-HSFIKQEPSWGGAEPEHEEQCLSAFTVHFSGQFTGTAGACRYGPFPPPPS	118
Mouse	PPPPP--PPPHSFIKQEPSWGGAEPEHEEQCLSAFTLHFSGQFTGTAGACRYGPFPPPPS *****.*****.*****.*****.*****.*****.*****.*****.*****.*****	118
Dog	QASSGQARMFPNAPYLPSCLESQPAIRNQAGYSTVTFDGTGPSYGHTPSHHAAQFPNHSFK	179
Human	QASSGQARMFPNAPYLPSCLESQPAIRNQ-YSTVTFDGTGPSYGHTPSHHAAQFPNHSFK	177
Mouse	QASSGQARMFPNAPYLPSCLESQPTIRNQG-YSTVTFDGAPSYGHTPSHHAAQFPNHSFK *****.*****.*****.*****.*****.*****.*****.*****.*****.*****	177
Dog	HEDPMGQQGSLGEQQYSVPPVYGCHTPTDSTGSAQALLLRTPYSSDNLQMTSQLECMT	239
Human	HEDPMGQQGSLGEQQYSVPPVYGCHTPTDSTGSAQALLLRTPYSSDNLQMTSQLECMT	237
Mouse	HEDPMGQQGSLGEQQYSVPPVYGCHTPTDSTGSAQALLLRTPYSSDNLQMTSQLECMT *****.*****.*****.*****.*****.*****.*****.*****.*****.*****	237
Dog	WNQMNLGATLKGVAAGSSSVKWTGEGQSNHGTGYESDNHTTPILCGAQYRIHTHGVFRGI	299
Human	WNQMNLGATLKGVAAGSSSVKWTGEGQSNHSTGYESDNHTTPILCGAQYRIHTHGVFRGI	297
Mouse	WNQMNLGATLKGMAAGSSSVKWTGEGQSNHIGIYSENHTAPILCGAQYRIHTHGVFRGI *****.*****.*****.*****.*****.*****.*****.*****.*****.*****	297
Dog	QDVRVPGVAPTLVRSASETSEKRPFMCAYPGCNKRYFKLSHLQMHSRKHTGEKPYQCDF	359
Human	QDVRVPGVAPTLVRSASETSEKRPFMCAYPGCNKRYFKLSHLQMHSRKHTGEKPYQCDF	357
Mouse	QDVRVSGVAPTLVRSASETSEKRPFMCAYPGCNKRYFKLSHLQMHSRKHTGEKPYQCDF *****.*****.*****.*****.*****.*****.*****.*****.*****.*****	357
Dog	KDCERRFSRSQDKRHRHTGVKPFQCKTCQRKFSRSDHLKTHTRHTGKTSEKPFSCR	419
Human	KDCERRFSRSQDKRHRHTGVKPFQCKTCQRKFSRSDHLKTHTRHTGKTSEKPFSCR	417
Mouse	KDCERRFSRSQDKRHRHTGVKPFQCKTCQRKFSRSDHLKTHTRHTGKTSEKPFSCR *****.*****.*****.*****.*****.*****.*****.*****.*****.*****	417
Dog	WPSCQKKFARSDELVRHHNMHQRNMTKLQAL	451
Human	WPSCQKKFARSDELVRHHNMHQRNMTKLQAL	449
Mouse	WHSCQKKFARSDELVRHHNMHQRNMTKLHVAL * *****.*****.*****.*****.*****.*****.*****.*****.*****	449

**Fig. 2.** Comparison of the predicted WT1 amino acid sequences among different species. The amino acid sequence of cWT1 was aligned with its human and mouse counterpart using the “Clustal W” software. “\*” indicates that the residue was identical, whereas “:” and “.” indicate that very similar and similar residues in the alignment were observed. The amino acid sequences of human and mouse WT1 were obtained from the NCBI database (X51630.1 for human and M55512.1 for mouse).

information in future investigations of the cWT1 expression pattern in canine tumors.

The main objective of this study was to investigate the relevance of cWT1 as a tumor associated antigen (TAA) in canine malignancies and its applicability as one of the immunotherapeutic options in the near future. Many TAAs have been identified in various human malignancies, and among them, WT1 was found to be commonly expressed [3]. Drakos *et al.* evaluated the expression of WT1 in 167 non-Hodgkin’s lymphoma (NHL) samples using IHC and found that 80% of Burkitt lymphomas, 75% of ALK-positive ALCLs, 50% of lymphoblastic lymphomas, 45% of ALK-negative ALCLs, 33% of DLBCLs and 17% of cutaneous ALCLs were positive for WT1 expression [4]. Since lymphoma is the most common hematopoietic malignancy in dogs, we focused on the expression of cWT1 in canine lymphoma. In this study, we revealed that cWT1 expression was detected in all of the canine lymphoma tissue specimens, including low and high grades, as well as both T and B cell lymphomas.

The IHC results have shown that cWT1 expression was detected not only in the lymphoma tissue specimens, but also in some normal tissue specimens. This raises concerns, if cWT1 is used as a TAA for immunotherapy, the possibilities of tolerance to treatment. The expression of WT1 protein in normal tissues is not an exclusive phenomenon in dogs and has also been reported in various human adult tissues, including tissues of the urogenital system, central nervous system and hematopoietic system, such as bone marrow and lymph nodes [11]. Nonetheless, the results of several clinical trials have shown that WT1 peptide vaccines in cancer patients are capable of eliciting immune responses with minimal adverse events [1, 9]. This suggests that even though WT1 is expressed in normal tissues, treatment tolerance will not be induced. In this study, expression of cWT1 was detected in a wide range of normal tissues as compared to that in human. This indicates that more prudent evaluations will be required in order to predict the autoaggression of the immune response against cWT1 in dogs.



**Fig. 3.** Cross-reactivity of the anti-human WT1 antibody with cWT1 overexpressed in HEK293T cells. HEK293T cells were transiently transfected with pcDNA3.1 (+)-cWT1 or pcDNA3.1 (+). Forty-eight hours after transfection, whole cell lysates were extracted, and Western blotting using anti-human WT1 antibody (6F-H2) was performed. Arrow indicates a band with the expected molecular weight of cWT1.

**Table 1.** Results of the immunohistochemical staining of normal canine tissues

Tissue	n	WT1 expression	Stained cells
Heart	2	–	
Spleen	2	–	
Kidney	3	+	Podocytes
Testis	2	+	Seminiferous tubular epithelium
Prostate gland	2	–	
Skeletal muscle	2	–	
Skin	1	–	
Lymph node	12	+	Lymphocytes

n, number of samples; +, positive staining; –, negative staining.

**Table 2.** Results of the immunohistochemical staining of lymphoma tissues

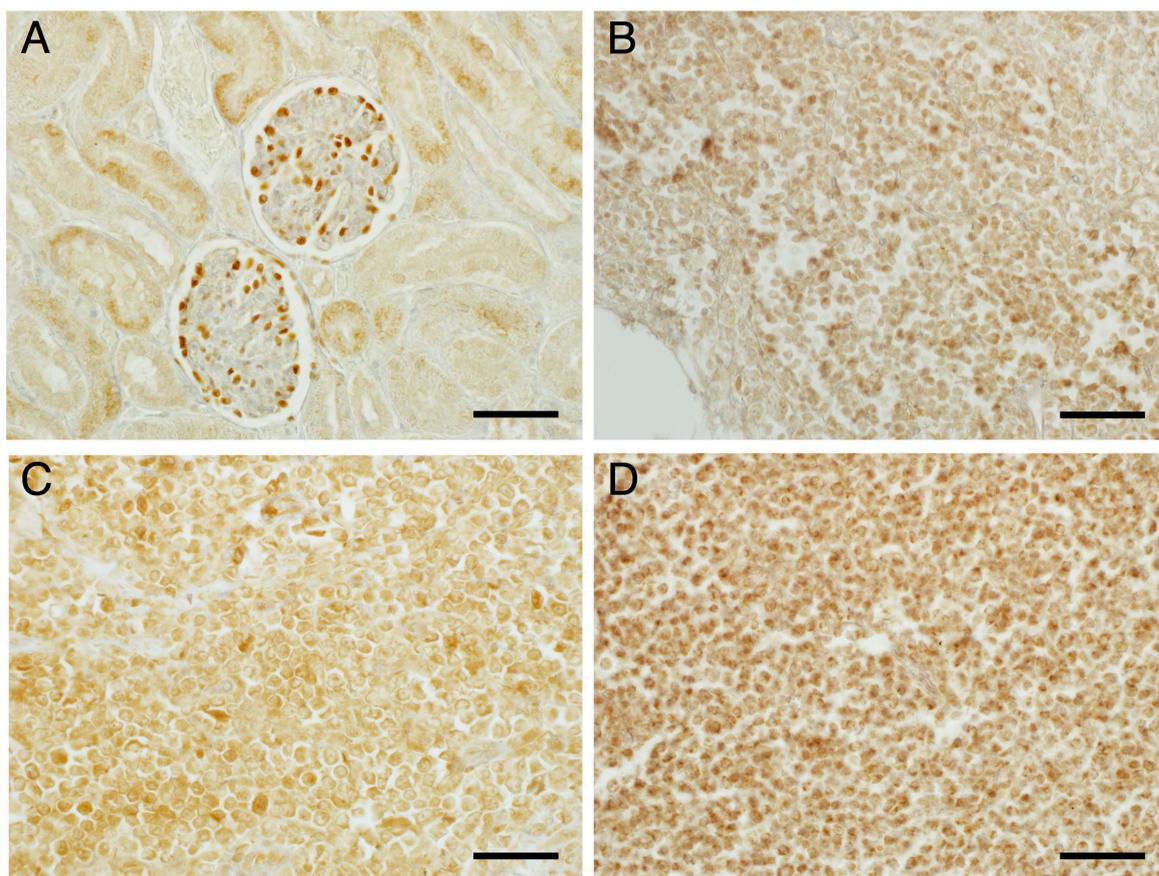
Immunophenotype	Grade	Anatomic site			
		Multicentric	Alimentary	Mediastrial	Extranodular
B cell	High	1	4	1	0
	Low	1	1	0	2
T cell	High	3	3	0	0
	Low	5	1	0	0

Identification of TAAs for use in cancer immunotherapy for canine lymphoma is of increasing importance. Latest therapeutic advancements in veterinary oncology include cancer vaccines, where treatment efficacy of human tyrosinase was evaluated in advanced canine melanoma and telomerase in B cell canine lymphoma [10, 18]. These studies indicate that cancer immunotherapy designed based on appropriate TAAs will exert clinical effectiveness. Therefore, we hope that more studies can be conducted to elucidate the therapeutic potential of cWT1 in canine lymphoma and to reveal cWT1 expression in other canine malignancies.

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**Fig. 4.** Representative results of cWT1 immunohistochemical staining and counterstaining with hematoxylin in normal canine tissue specimens and lymphoma tissue specimens. (A) Normal kidney, (B) normal lymph node, (C) high grade T cell lymphoma from the intraperitoneal lymph node and (D) high grade B cell lymphoma from the spleen were stained with 6F-H2. Scale bar indicates 50  $\mu$ m.

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