Swedish University of Agricultural Sciences
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Title: Immunoassays for detection of serum Thymidine kinase 1 in Dog lymphomas and carcinomas

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ABSTRACT:

Serum thymidine kinase 1 (TK1) activity determination is used as marker for tumor monitoring in both human and veterinary medicine. TK1 is an intracellular enzyme involved in the salvage pathway of DNA precursor synthesis. TK1 expression is cell cycle dependent and the activity increases markedly at the end of G1 and reaches a peak value in S phase and then declines rapidly in G2 /M. The pronounced proliferation in tumor cells result in a higher TK1 activity within cell. So determination of TK1 activity in tumors provides information regarding prognosis and effectiveness of treatment. In human medicine, a labeled radioactive substrate is used for measuring TK1 activity. Some studies have shown that this method can be used also in veterinary medicine. However, it has limitation for general use in animal practice since it is expensive and involves handling of radioactive material. In human medicine, anti TK1 antibodies have been used to determine the concentration of serum TK1 in patients with carcinomas. This type of measurements has been successful and would be valuable to translate into veterinary medicine. The purpose of this study is to make the use of anti-dog TK1 antibodies for detection of serum TK1. Anti bodies have been produced by immunizing the rabbit with synthetic piece of a dog TK1 peptide. Suspected band of serum TK1 has been found in the serum from dogs with tumors but not in the serum from healthy dogs using these antibodies. This study showed that anti dog TK1 polyclonal antibodies produced by affinity chromatography binds to serum TK1 with adequate capacity. It has been also shown that these antibodies can bind with both human and dog cytosolic TK1. These results are the first step in the development of an ELISA, which may provide clinical information about prognosis, risk of recurrence and effectiveness of antitumor therapy in veterinary medicine.

Background to the Veterinary medical problem:

Dogs are prone to various neoplastic diseases, like lymphomas, mammary tumors and leukemia's. When lymphocytes undergo transformation into cancerous cells, invasion of these cells into bone marrow and other organs (like spleen, thymus) of lymphatic system and forms solid tumors within these organs are called lymphomas (lympho sarcomas). They may originate from T-cells or B-cells. Lymphomas are the most common form of cancer in dogs; the annual incidence has been estimated as 13 to 40 cases per 100,000 dogs. Lymphoma accounts for 7-24 % of all canine neoplasia. Dog breeds with high incidence of lymphomas includes Boxers, Bull Mastiffs, Basset Hounds and Scottish terrier; breeds with a low risk include Dachshunds and Pomeranians. Lymphomas are more commonly seen in 6-9 years old dogs and the etiology of the lymphomas is unknown and most likely multi factorial (Lane *et al*, 2007).

The Canine lymphoma staging system can be summarized as follows: lymphomas are staged with WHO scheme according to nodal involvement and divided into stages I, II, III, and IV and they are further sub-classified into a) asymptomatic b) sick; followed by indicating the organ involvement (hepatic, splenic and bone marrow). Dogs that are sick with involvement of organs should be considered as clinically advanced stage.

Table 1. Clinical stages of canine lymphoma

I.	Involvement limited to a single node or lymphoid tissue in a single organ.
II.	Regional involvement of many lymph nodes with or without involvement of tonsils.
III.	Generalized lymph node involvement,
IV.	Liver and\ or spleen involvement (with or without stage III).
V.	Manifestation in the blood and involvement of bone marrow and\or other organ system(s).

Each stage is sub classified into A (with systemic signs) and B (without systemic signs).

Clinical observations:

The clinical observations mainly depend on the extent and location of tumor. Multi centric lymphoma is the most common form with generalized lymphadenopathy, hepatosplenomegaly and most of the dogs are asymptomatic but nonspecific signs like anorexia, vomiting, diarrhea, asicites and fever are common. In alimentary lymphoma gastro-intestinal signs such as vomiting, diarrhea, weight loss, and malabsorption are common. Mesenteric lymph nodes, spleen and liver may be involved. The mediastinal form of lymphomas is characterized by enlargement of the craniomediastinal structures or thymus. Respiratory distress, exercise intolerance, polyuria from hypocalcaemia are frequently observed. Cutaneous lymphoma is usually generalized and skin becomes more erythemtous, thickened, ulcerative and exudative. Sometimes oral involvement also occurs (Lane *et al*, 2007).

Diagnosis:

Physical examination of lymph nodes, complete blood picture, biopsy and microscopic examination are required for an accurate diagnosis in the early stages of lymphomas. Fine needle aspiration and radiographic examination are useful in different stages of lymphomas. Malignant lymphomas cannot be cured completely, however, early detection and efficient chemotherapy can control the disease for long time.

Prognosis:

The prognosis of canine lymphoma depends on various factors like location of tumor, extent of the disease, presence and absence of clinical signs. Immunophenotyping of the lymphomas into B- cell and T- cell type may assist in the prognosis. Another prognostic indicator involves the use of cell proliferation markers in tissue specimens. Several attempts have been made to use these markers for prognosis and relapse of the lymphomas in dogs. Serum alpha 1 acid glycoprotein concentration was used for predicting the relapse of malignant lymphoma. Alpha-fetoprotein can be a good marker in advanced stages of malignant lymphomas, where the concentration of this protein is high (von Euler *et al*, 2004).

Biomarkers in Veterinary Medicine:

Cell proliferation markers commonly used in veterinary medicine are Ki-67, argyrophilic nuclear organizing regions (AgNOR), Mitotic index, proliferation cell nuclear antigen (PCNA), lactate dehydrogenase (LDH) (Kiupel *et al,* 1999).

Monoclonal antibody, Ki-67 recognizes a nuclear antigen present in proliferating cells and permits an immunohistochemical method of assessing tumor cell proliferative fraction. It is expressed in all phases of active cell cycle (G1, S, G2 and M) and it is absent in resting cells (G0) (Leong *et al*, 1995). AgNOR is a good marker for the prognosis of lymphoma. The quantity of the AgNOR not only reflects the percentage of cells in cycle but it also increases when the cell cycle is faster and AgNOR counts correlates well with the tumor grade (Kiupel *et al,* 1998). Studies on AgNOR demonstrated significant predictive potential for remission in treated and untreated cases of canine lymphoma (kiupel *et al,* 1999). However, histopathological examination of AgNOR does not help for disease monitoring. AgNOR is associated with problems in interpretation and is dependent on type of the fixation method, temperature and staining time. Mitotic index is an indirect measure based on quantification of the proportion of mitotic cells in a histopathologic specimen and it is easier to perform than AgNOR and Ki-67 assessment (Romansik *et al*, 2007). PCNA is produced in late G1 phase and throughout S-phase, but it can be detected in all proliferating cells because of long half life, High concentrations of PCNA are detected in S phase. It has been used to predict prognosis in melanomas in dogs and cats (Roels *et al,* 1999). In one study, plasma lactate dehydrogenase (LDH) was used for evaluation of disease progression in malignant lymphoma of dogs (Nakamura *et al*, 1997). They found elevated levels of plasma LDH in tumors and in some hemolytic diseases. In certain serum samples of dogs with tumors, they did not find any increase in the plasma LDH level. Furthermore, the sensitivity and specificity of LDH is very low (Nakamura *et al*, 1997). Thymidine kinase 1 expression both at the RNA and protein level has been used extensively as a marker for proliferating cells and TK1 is one of the protein, whose abundance is most closely correlated to the S-phase. Because of its association with S phase, it is a good proliferative marker for monitoring and prognosis of tumor diseases.

Introduction to the project:

Thymidine kinase (TK) is a cytosolic enzyme involved in the pyrimidine salvage pathway, which are building blocks of DNA. Thymidine kinase catalyzes the conversion of deoxythymidine to deoxythymidine monophosphate (dTMP) in the presence of ATP by transferring the γ- phosphate group. This phosphorylation step is often a rate-limiting step and deoxythymidine monophosphate is subsequently phosphorlyated to di and triphosphates before serving as a substrate for DNA synthesis. TK enzymes in mammalian cells appear in two forms, a cytosolic thymidine kinase (TK1) and the mitochondrial (TK2) form. Thymidine kinase 1 (TK1) is present in the cell cytoplasm and is involved in cell proliferation. TK2 is involved in mitochondrial DNA synthesis, which is independent of the cell cycle (von Euler *et al*, 2006).

TK1 plays a primary role in regulation of intracellular thymidine nucleotide pools during the cell cycle. The level of TK1 increases from late G1 to late S phase and early G2 phase in both proliferating and tumor cells. Thymidine kinase may also appear in the body fluids because of cells with disrupted spindle apparatus (von Euler *et al*, 2004). TK1 or TK1 like genes seems to be widely distributed in many organisms from plants to humans, including bacteria and viruses (Welin *et al*, 2004). The human TK1 gene is 12.9 kb with seven exons. The 1241bp cDNA with an open reading frame of 702 bp, encoding a 234 amino acids long protein with a molecular weight of 25.5 kDa and TK1 gene is located on chromosome 17 (Eriksson *et al*, 2002). To characterize serum TK1 reducing agent treatment were used and only high concentration of DTE (Dithioerythritol, 400 mM) showed effects on the molecular forms of TK1 going from more than 700 kDa to about 100 kDa (Karlström *et al,* 1990). In human medicine TK1 activity is measured by using a commercially available assay; the Prolofigen TK-REA (radio enzymatic assay), where a radioactive substrate analogue is added. It then measures the amount of converted substrate per unit time, which represents the TK activity in serum.

In one study, they used the same method to determine the TK1 activity in the serum of dogs with malignant lymphoma and non-hematological or healthy ones (von Euler *et al*, 2004). Serum TK1 activity was significantly higher in lymphoma than in normal dogs. Average TK1 activity after complete remission was not significantly different compared with healthy dogs. Thus, measuring TK1 activity in dog’s serum serves as a

tumor marker providing information regarding prognosis and risk of relapse in dogs with tumors (von Euler *et al,* 2004). One publication has described the use of plasma thymidine kinase activity in diagnosis and monitoring of lymphomas and leukemia in dogs (Nakamura *et al*, 1997).

In human medicine, serum TK1 activity has been used for estimating the extent of tumor disease and information of the prognosis of leukemia's, multiple myelomas, Hodgkin's lymphoma and non-Hodgkin's lymphomas (Wu *et al*, 2003). Serum TK1 activity has been determined by using commercially available TK-REA. This assay provides prognostic information of malignancies and some cases it can aid in the choice of therapy (Topolcan *et al,* 2008). A non-radiometric TK enzyme linked immunosorbent assay (ELISA) was developed by using 3'-azido-2'-deoxythymidine (AZT) as substrate, which is phosphorylated selectively by TK1 in the serum. Furthermore, a competitive ELISA measures the amount of AZT monophosphate using goat polyclonal antibodies (Öhrvik *et al*, 2004). Recently, a Liaison assay was developed. It is a non-radiometric assay and technically more accurate, reliable in measuring the TK1 activity in dogs with lymphomas. In brief, TK1 in the tumor serum sample catalyzes phosporylation of 3'-azido-thymidine (AZT) to the corresponding 5'- monophosphate(AZTMP). In the second step, the produced AZTMP competes with AZTMP conjugated to isoluminol(AZTMP-ABEI) for specific antibodies bound to magnetic beads. Free AZTMP-ABEI is removed by washing and the amount of bound AZTMP-ABEI is determined by chemiluminescence (von Euler *et al ,* 2008).

The Biovica Company Uppsala (Sweden) has developed the very sensitive Divi Tum assay, which is a non-isotopic method and uses bromo-deoxyuridine as substrate for TK followed by its incorporation into an oligonucleotide attached to a solid support (refer www.biovica.com). Even though we have, many assays to determine the serum TK1 activity in dogs most of them needs special equipment that are expensive. The dog TK1 gene (amino acid) sequence shows 88.5% similarity with the human. TK1 sequence diversity is 9.1% and most of the differences in sequence is seen in the C-terminal part (Fig 1)

Dog: AYTKRLGSEK EVEVIGGADKYHSVCRLCYFKKASGPPMGLDSERNKENVL 210
HUMAN: AYTKRLGTEKEVEVIGGADKYHSVCRLCYFKKASGQPAGPDNKEN----- 210

DOG: VLVPGKPGEGKEATGVRKLFAPQHVLQCSPAN 243
HUMAN: CPVPGKPGE------ AVAARKLFAPQQILQCSPAN 235

Fig 1: The sequence of Dog and Human TK1 (160-243) . Red color indicates the differences in the amino acid sequence. The underlined sequence amino acids (196-223) are used for immunization of rabbit.

The use of TK1 serum tests in humans has increased recently and most of these tests are based on activity measurement with radio labeled substrates. In veterinary practice, the commercially available tests are not a convenient alternative because of the need for a special instrument. Still the result with the Liaison TK assay serves as a good indicator for the value of this type of information for prognosis and monitoring of dogs with malignant lymphoma.

In human medicine anti human TK1 anti bodies have been utilized for measuring the serum TK1 levels in various conditions, e.g. Lung cancer, colorectal and mammary cancers (He *et al*, 2000). However, it has not been described by any method that makes use of dog TK1 antibodies. So it would be interesting to produce the dog TK1 antibodies and evaluate them further whether they can provide a simpler method for prognosis of tumors in dogs. Three different rabbits Ulrika (U), Dingo (D) and Kurtis (K) were immunized with synthetic peptide (amino acids 196-223) by Agri Sera AB (Umea Sweden). Anti sera were already collected from these rabbits after different periods of immunization before starting project.

The main purpose of this project is to purify these antisera using affinity chromatography, which was produced by coupling the C-terminal peptide shown in Fig 1 to a Sepharose 4B matrix. These purified antibodies are used to study serum TK1 and eventually determine its concentration in various serum samples. Serum TK1 activity measurements were determined with radioactive thymidine as substrate. Another objective of this work was to collect blood samples from dogs with tumor diseases and analyze TK1 levels, trying to find a correlation with disease condition and serum TK1 levels.

Materials and methods:

All serum samples were collected from dogs with tumors at the Department of clinical medicine, small animal clinic, SLU. Cell cultures from dog MDCK (madin Darby canine kidney cell lines) and the human T-lymphoblast cell lines like CEM TK^{+} and TK^{-} variants were also used.

Purification of rabbit anti TK1 antibodies by Sepharose coupled with C-terminal TK1 peptide:

CNBR-activated Sepharose 4B (1 gram in final volume of 3.5 ml) was treated with HCl to remove the preservatives and mixed with dog peptide i.e. ***(CPMGLDS RENKENVLVLVPGKPGEGKEANH2)*** at a concentration of 2 mg ⁄ml in 3 ml of coupling buffer (0.1 M NaHCO3 pH-8.3 containing 0.5 M NaCl). The coupled Sepharose was washed with five medium volumes of coupling buffer and remaining active groups were blocked by transferring the medium to 0.1 M Tris-HCl buffer, pH 8.0. The Sepharose was then treated with three cycles of alternating 0.1 M acetate buffer, pH 4.0 containing 0.5 M NaCl and 0.1 M Tris-HCl, pH 8.0. Finally, the column was washed with PBS. For purification of anti TK1 antibodies filtered 15 ml of rabbit anti-serum was diluted in PBS to 50 ml and loaded on the affinity column. It was washed with 20 ml (50 mM Tris-Cl, pH 8.0; 0.1% Triton X-100; 0.5 M NaCl) followed by (50 mM Tris-Cl, pH 9.0; 0.1% Triton X-100; 0.5 M NaCl), with 50 mM sodium phosphate, pH 6.3; 0.1% Triton X-100; 0.5 M NaCl. Elution of bound rabbit anti TK1 antibodies was performed with 20 ml 50 mM Glycine –HCl, pH 2.5; 0.1% Triton X-100; 0.15 M NaCl into tubes containing 4 ml of 1 M Tris-Cl, pH 9.0, to neutralize the samples.

Determining the protein concentration:

The protein concentrations were determined using the Bio-rad protein assay. The reference used here was BSA (bovine serum albumin) in PBS.

The Bio-rad reagent was diluted with water and four different concentrations of BSA (2.7, 5.4, 7.1 and 10.8 µg respectively) has made to set reference. Two samples of antibody solution were used. Absorbance was measured at 595 nm and a reference graph was created with the BSA standards. With the help of the graph, the concentration of antibody protein was determined.

Dot blot immuno assays:

The TK1 levels in serum samples were measured using the enhanced chemiluminscent (ECL) dot blot assay (He et al 2000). In this assay, 3 µl of serum is applied to the nitrocellulose membrane (Hybond TM-C, Amersham). Recombinant dog TK1 was used as standard. The membranes were left to dry and then blocked with 10 % non-fat milk in TBS for one hour. The membranes were incubated with primary antibody (1.25 µg ⁄ml) for two hours and washed with TBS-T (0.1% Tween 20) for 15 minutes. Secondary antibodies conjugated with HRP were added (2 ng ⁄ml) and agitated for one hour (He *et al,* 2000). Then the membranes were washed with TBS-T for 15 min and ECL substrate applied to membrane for 1 min, and then the membrane was exposed to X-ray film. The serum TK1 levels were determined by measuring the intensity of the spot and compared to a TK1 standard.

Depletion of albumin and IgG from the serum:

Proteoprep blue albumin and IgG depletion kit (SIGMA, USA) was used for depletion of albumin. This kit removes more than 85% albumin and 70% of IgG in the serum samples. It involves two steps a) column equilibration: medium slurry of 0.4 ml was transferred to a spin column. Storage solution was removed by centrifugation. Equilibration buffer (0.4 ml) was added to the spin columns and centrifuged. The buffer from collection tube was discarded after centrifugation and replaced with new collection tube. Then this column was used for serum depletion. b) Depletion of albumin and IgG from the serum samples: Serum samples of 100 µl were added to spin column and allowed for 10 minutes at room temperature. So that albumin and IgG in serum are bound to the slurry in the spin column. Centrifugation and reloading of the elution to the same spin columns removed an additional 5% of the albumin. The depleted serum in collecting tube was used for further evaluation.

Isolation of serum TK1 using the anti TK1 antibody Sepharose:

Serum samples 100 µl from healthy dogs and dogs with tumors after depletion of albumin were diluted with 100 µl of TBS and incubated with the anti dog TK1 antibody Sepharose (200 µl). The samples then agitated at 4°C for 3 hours followed by centrifugation. The sepharose was washed two times with TBS, one time with TBS-T and one time with TBS. Then 40 µl of sample buffer (containing 0.5 M Tris-HCl, pH 6.8, glycerol, 10% (w\v) SDS, and 0.1% bromophenol blue) was added to

the Sepharose and incubated at room temperature for 30 minutes to elute bound serum proteins. These samples were heated at 95°C for 5 minutes and analyzed by SDS-PAGE.

Western blot:

SDS-PAGE and electrophoretic transfer was done as described in Amersham life sciences western blotting protocols. Samples were loaded on the gel and run for 1 hour at 160 V and 6 µl of marker (Fermentas, USA) for reference protein molecular weights. Proteins were transferred to PVDF membranes (GE HealthCare) at 90 V for 50 min. Membrane was rinsed 3×5 min with TBS buffer (pH 7.6) and blocked in TBS-T (5% BSA) buffer. The membrane was incubated with primary antibody (1.25 µg ⁄ml) and then incubated with goat-Anti rabbit F (ab)$_2$ fragment and avidin HRP conjugated (Jackson, USA). The membrane was briefly rinsed with TBST buffer twice, and then washed once for 15 min and twice for 5 min with TBST buffer. The membrane was incubated with the ECL reagents (GE HealthCare) for precisely 1 min at room temperature and then placed in film cassette for 2-5 min exposure (hyper film-ECL).

Immunoprecipitation:

Immnuoprecipitation with Protein-G sepharose was done as described by (GE HealthCare). Serum samples of 200 µl along with buffer (containing TBS, pH 7.6, 1 mM befa block, 1% BSA and purified anti dog TK1 antibodies of 2µg ⁄ 500µl) to make final volume of 500µl but in cell extract the protein concentration was 500µg. These tubes incubated at 4°C overnight. Then the samples were mixed with 100µl of Protein-G sepharose. These tubes were kept at 4°C with slight agitation for 4 hours. The Sepharose beads were centrifuged and washed with TBS two times, one time with TBS-T and then with TBS. Protein-G sepharose was incubated with sample buffer (0.5M Tris-HCl, pH 6.8, glycerol, 10% (w\v) SDS, 0.1% bromophenol blue at room temperature for 20 minutes to elute the antibody complexes. The samples were analyzed by western blotting as described.

Determination of enzyme activity:

Enzyme activity of the serum samples was determined by using radio labeled ^{3}H-thymidine as a substrate. The reaction was started by adding 20 µl TK assay buffer (0.1 M Tris-HCl, 0.15 M NaCl, 5 mM $MgCl_2$, 5 mM NaF, 2 mM DTT, and 5 mM ATP) to 20 µl of serum samples. The substrate was phosporylated by enzyme in serum with ATP as phosphate donor. The reaction was interrupted at different time

points (30, 60, 120 and 240 min) to measure the amount of product formed. A small fraction (10 µl) of reaction mixture was transferred to DE-81 paper disks. These disks were washed two times with 1mM ammonium formate for 10 minutes of each. Then paper disks were incubated in 1 ml of (0.1 M HCl and 0.2M KCl) for 45 minutes to elute the product which were measured by scintillation counting. The results are expressed in pmol ⁄ min.

FPLC (fast protein liquid chromatography):

FPLC was used for rapid separation of proteins from complex mixtures. Sera and cell extracts (100 µl) were diluted two fold with buffer (0.01 M Hepes pH -7.6, containing 0.15 M NH_4Cl and NaN_3) and then filtered. 200 µl samples were fractioned by sepharose 12 chromatography (GE HealthCare) in a column of 1.0×30 cm. Protein content was monitored continuously at 280 nm. The column was eluted with the same buffer and 350 µl of 24 fractions were collected. 200 µl of these fractions are precipitated with 10% TCA and did western blot as described above. Four proteins; alpha 2 –macro-globulin, BSA, Ova albumin and Horse myoglobulin with the molecular weights of 700, 66 ,45 and 17 kDa were used as molecular weight markers.

Results:

Sera were collected from rabbits 4 weeks after immunization. Anti serum was loaded on affinity column made with the C terminal region of dog TK1. The bound anti TK1 antibodies from rabbit (D) and rabbit (U) were purified in this way. The concentration of antibodies was determined by the Bio-rad assay. Protein concentration in case of the rabbit (D) antibodies was 0.8 mg/ml and from rabbit (U) 0.5 mg/ml. The purified antibodies from D (Fig 2) were tested with human recombinant TK1 (234 aa + his tag) and dog recombinant TK1 Δ12 (230 aa + his tag) (see appendix)

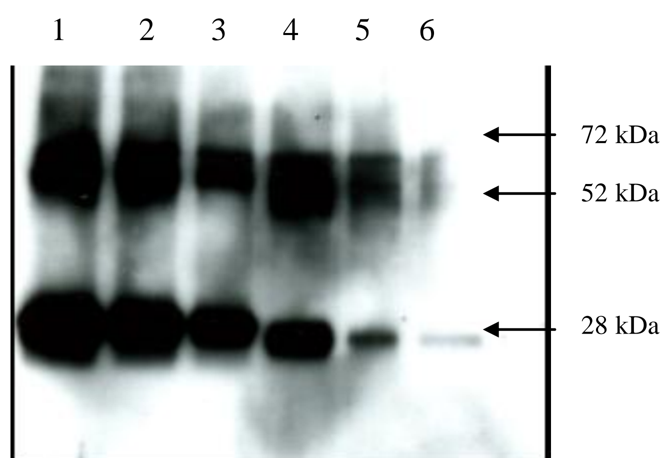

Fig 2: Western blot analysis with purified rabbit (D) antibody (1.25 μg /ml) with human and dog recombinant TK1. Lane 1-3 are dog recombinant TK1Δ12 (2, 1 and 0.5 ng); 4-6 are human recombinant TK1 (2, 1 and 0.5 ng).

Normally TK1 exits as dimer and by activation with ATP (adenosine triphospate) it converts into tetramer. The sub unit of TK1 in lower region at 28 kDa in case of both human and dog (Fig 2) (see appendix page 29 and 30). In Fig 2, an apparent dimeric form of TK1 is seen as strong band between the 52 kDa and 72 kDa. To analyze the upper band of TK1, mass spectrometry of the bands in that region was performed. The band was conformed as TK1 , which corresponds to molecular weight of 66 kDa (approx). peptide analysis results were shown in appendix (page 32).

The anti dog TK1 antibodies from D reacts with cytosolic TK1 in crude cell extracts from dog MDCK cells but also with human TK1 in CEM TK^{+} cells but no band was seen in CEM TK^{-} cell extracts (Fig 3).

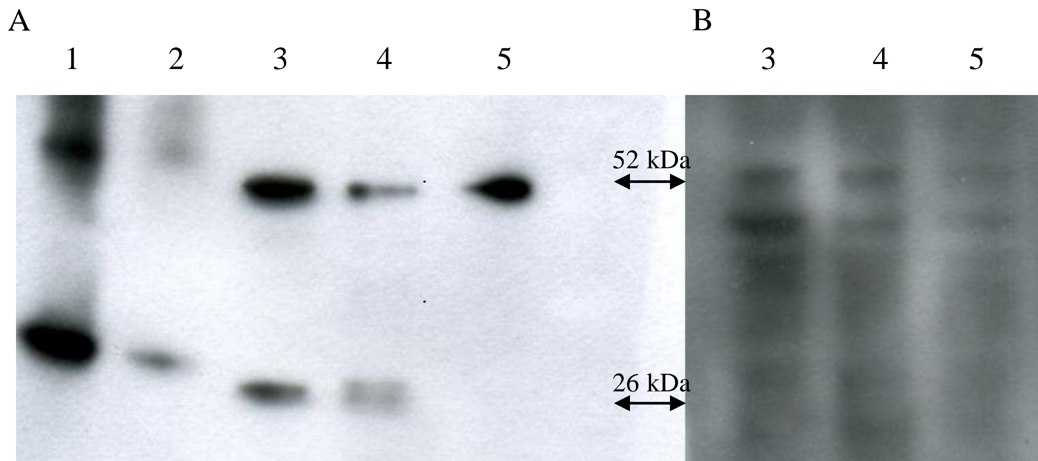

Fig 3: Western blot analysis with TK1 antibody from D (1.25 µg/ml) the absence (A) and in presence (B) of an excess of the antigen peptide (0.1 µg/ml). Lane 1, 2 are Dog recombinant TK1 (0.25, 0.05 ng) 3, 4, 5 are extracts from cell lines (CEM TK^{+}, MDCK, CEM TK^{-} approx 100 µg protein in each case).

The band at 26 kDa, which was found in both CEM TK^{+} and MDCK cell extracts corresponds to cytosolic TK1 and the band was absent in CEM TK^{-} cell extract. MDCK extract from a cell culture grown to high density cell lines were tested and in this case a double band was found in the region corresponding to cytosolic TK1. There were no bands on membrane B in lower TK1 band region demonstrating that an excess of peptide could block the reactivity with all the polypeptides seen in fig 3A. The band that appeared at 52 kDa is most likely not related to TK1 since this band was observed in extracts from TK^{-} cells and also on the membrane B.

Anti dog TK1 antibodies from U and D were used to test the reactivity with human and dog recombinant TK1 and cell extracts.

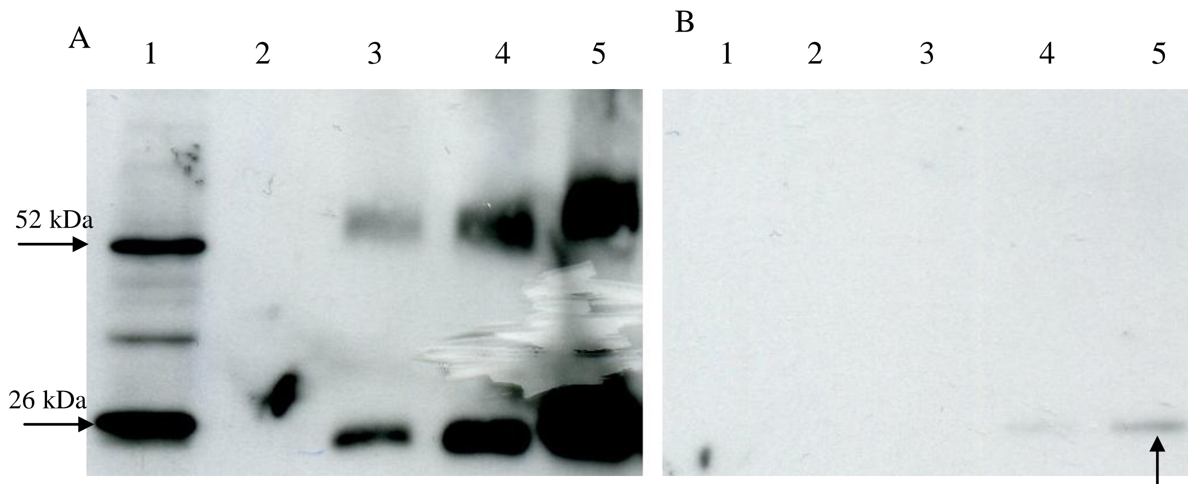

Fig 4: Western blot analysis with anti dog TK1 antibodies from U (A) and D (B). Lane 1 is recombinant dog TK1 (0.5 ng) 2, 3 are human TK1 (2 and 1 ng) 4 is MDCK cell extracts from high density cultures, 5 is MDCK cell extract from exponentially growing cells and a total of 25 μg protein was tested.

The membrane with antibodies from rabbit U has shown light band with dog recombinant TK1 (indicated by arrow) and no reactivity with cytosolic TK1 from dog cell extracts (Fig 4A). Anti bodies from rabbit D showed good reactivity with both dog and human recombinant TK1 (Fig 4B). the sub unit of cytosolic TK1 (26 kDa) was appeared as single in MDCK extract from exponentially growing cells. This was different as double band was appeared in this region in MDCK cell extract from high dense culture(Fig 3A).

Based on results from Fig 4B anti bodies from rabbit D were used for evaluation of TK1 from serum samples (serum from healthy dog and dogs with tumor disease). D anti bodies were coupled to Sepharose 4 B. Albumin was depleted from the serum samples before incubating with D anti TK1 anti body sepharose.

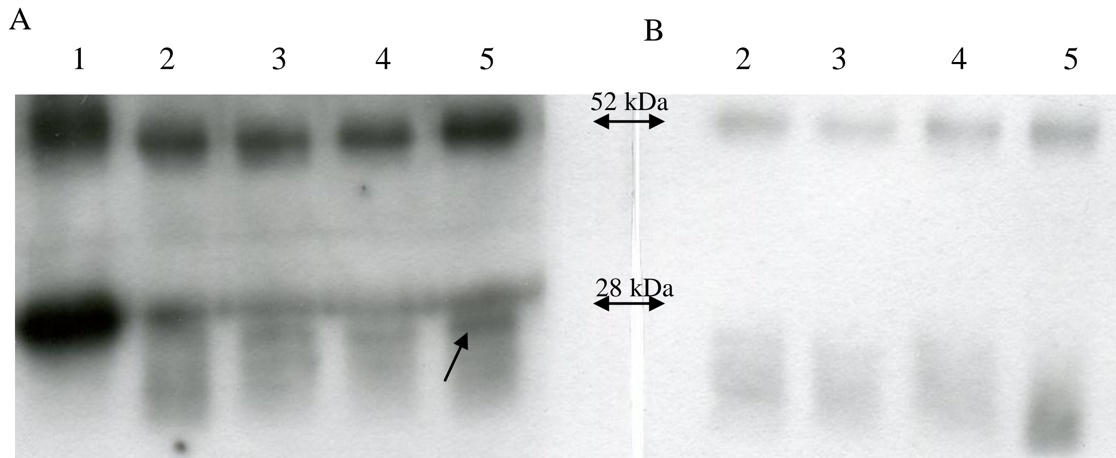

Fig 5: Western blot analysis of serum samples purified with D antibody Sepharose. Lane 1 is recombinant dog TK1 (0.5 ng), 2 is serum from a healthy dog and 3, 4, 5 are serum from dogs with tumor diseases. There were several bands observed on the membrane where no primary antibody was added, most likely because of cross reactivity to some serum proteins by the secondary anti rabbit antibodies.

In Fig 5A there was clear band just below recombinant TK1 (indicated by arrow) at a molecular weight of 28 kDa in sera from dogs with tumor diseased but not in sera from healthy dogs. This band was not seen in Fig 5B, and could therefore represent a form of serum TK1. The recombinant TK1 contains his tag protein (1.5 kDa) which may explain why it has a higher molecular weight than the presumed serum TK1 (appendix page 32).

Effects of different reducing agents on serum TK1: Reducing agents like DTT (dithiothreitol) or DTE at different concentrations were used because in earlier study (Karlström *et al*, 1990) it was observed that the molecular weight of TK1 was affected by these reducing agents in FPLC gel filtration analysis. Here we performed similar analysis but serum samples treated with reducing agents and performed western blot (up to 100 mM). This had no clear-cut influence on the apparent gel filtration pattern of TK1 with regard to high molecular and low molecular weight forms and that was different from what was found previously. However, in this case we did not use the very high DTE concentration (400 mM) which was done in the study by Karlström et al.

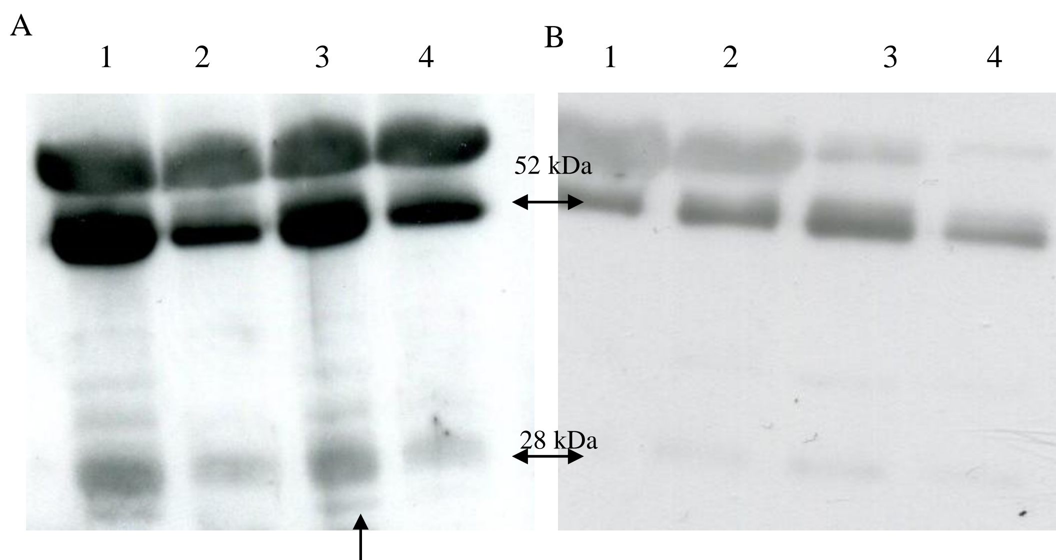

Fig 6: Western blot analysis of serum samples treated with reducing agents. In the figure 6, 1 and 2 are sera treated with 200 mM of DTT and 3, 4 with 200 mM of DTE. 1, 3 are sera from dog with tumor disease and 2, 4 are sera from healthy dogs.

In A the membrane is incubated with purified D antibodies and B is without of primary antibody. Suspected TK1 band appeared below the molecular weight of 28 kDa in sera from dogs with tumor disease (indicated by arrow) but not in sera from healthy dogs (see Fig 5A). The strong bands in the upper region are mainly due to cross reactivity of secondary antibodies with some serum proteins. Furthermore these bands were seen on the membrane without primary antibodies (Fig 6B). Reducing agents treatment was helpful in reduction of many unspecific proteins that appeared on membrane with direct serum samples.

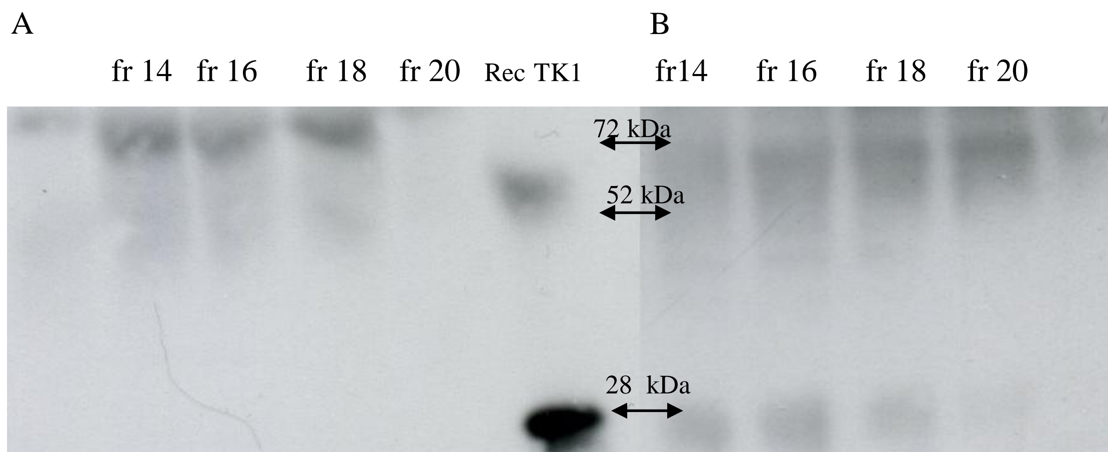

Fig 7. Western blot analysis of different FPLC fractions (fr) .In the Fig 7 , different FPLC fractions (14,16, 18 and 20) of both healthy and tumor diseased dog serum samples treated with 10% TCA (trichloroacetic acid) and recombinant dog TK1 of 0.5 ng. Membrane A is with FPLC fractions of healthy dog serum incubated with antibodies from D. Membrane is with tumor diseased dog serum sample FPLC fractions incubated with antibodies from D.

In FPLC fractions of healthy dog serum samples (Fig 7A) no bands were observed around the molecular weight of 28 kDa but in the case of tumor diseased serum fractions suspected band of TK1 sub unit was detected. In the (Fig 7B) both dimer (14 and 16 fractions) and monomer (fraction 20) forms of serum TK1 were identified.

Discussion:

The peptide sequence used to immunize rabbits was from amino acids 196-223, which is the C-terminal part and most variable region in human and dog TK1 sequence. The possible explanation for differences in reactivity of these two anti TK1 antibodies could be its epitopes. The epitope of D antibodies may present in both the human and dog amino acid sequence, since it was able to recognize both human and dog TK1. However, the epitope for the U antibodies is most likely found only in dog TK1. These U antibodies does not recognize the human recombinant TK1 at low concentration (eg.1 ng).

In serum samples, there was no clear cut bands observed with the U antibodies and some un specific bands found in the membrane without the primary antibody due to cross reactivity of secondary antibodies with serum proteins. Sometimes these antibodies may detect another compound than TK1 or may cross-react with other compounds than TK1. Furthermore modifications of serum TK1 will most likely alter the reactivity of different antibodies. It is known that TK1 found in a stable multimeric form in serum.

Human recombinant TK1 (28.2 kDa) has almost similar molecular weight as a truncated form of dog recombinant TK1 Δ12 (27.9 kDa). The dimeric form of dog recombinant TK1 was identified between 52-72 kDa, which was confirmed by peptide analysis (see page 32). The suspected band of serum TK1 sub unit was identified at 26 kDa and it had a molecular weight lower than recombinant TK1 due to His tag protein in recombinant TK1. The suspected TK1 band was found only in serum from dogs with tumor disease and not in serum from healthy dogs. Reducing agents like DTT and DTE up to 100 mM concentration did not have clear-cut influence on the serum TK1 band. However, with 200 mM concentration of DTE and DTT there was reduction in the other unspecific bands in the serum. FPLC gel filtration of serum samples on western blot analysis with antibodies from D have identified both dimer and monomer forms of TK1 in tumor diseased serum but not in serum samples from healthy dogs.

Some difficulty was found in obtaining dog cell extracts with sufficient TK1 levels to evaluate the function of these antibodies. In one experiment a double band of TK1 was found in extracts from MDCK cells grown to high cell density. However, in extracts from exponentially growing MDCK cells only one band with a molecular

weight corresponding to the human cellular TK1 was observed. There would be some degradation or phosporylation of cellular TK1, which may responsible for double band appearance in dog cell extract. Depletion of albumin from the sera reduced some unspecific bands and pre-treatment of serum with anti TK1 antibody removed many unspecific bands. This may permit the identification of the TK1 polypeptide in serum. Here we identified a protein band of molecular weight at 26 kDa in the serum of dogs with tumors, which may be TK1 and this is a major finding in this study.

Immunoprecipation of serum samples and cell extracts with purified anti dog TK1 antibodies did not give clear-cut result. There were many unspecific bands in both normal and sera from dogs with tumors. One general concern about the antibody-based assays is that they may be less sensitive than assays based on enzyme activity because there is considerable amplification of the signal during the enzyme reaction. Therefore, immunologic assays may not be able to determine low concentrations of TK1 accurately in sera from healthy individuals. An established immunological assay, which measures low concentrations of TK1 from healthy serum, would be an alternative to activity measurement. In this project, purification of anti serum to isolate C-terminal specific anti dog TK1 antibodies and coupling them with Sepharose gave some promising results. A specific TK1 protein of molecular weight at 26 kDa was found in sera from dogs with tumors. However, much further work is needed to translate these results to clinically useful methods.

Tumor markers play an important role in diagnosing cancer in early stages. Even though we have many tumor markers in veterinary medicine but there are no specific markers that gives clear indications of early stage tumors, which is very important in veterinary medicine. In order to produce sensitive, robust and a clinically valuable diagnostic test we most likely need antibodies with high specificity that are targeted to different epitopes. Owners or veterinarians may not recognize dogs in early stages of tumor disease. With the availability of a new immunoassay, it may be possible to diagnose the tumors earlier and make treatment more effective. An immunoassay for dog lymphomas may be used as a comparative model for non-Hodgkin's lymphomas in humans. The results of this study show that it is possible to produce antibodies that can detect TK1 in serum samples from dogs with tumors. This together with results from previous studies may provide a cheap and efficient ELISA for malignant lymphoma in dogs which may become a reality in the future.

Future prospects:

Using the affinity purified antibodies from rabbit D an ELISA could be developed which measures the concentration of TK1 in serum of dogs with tumors. It may provide a cheap and convenient test for dogs with tumors. Furthermore, it can help veterinarians in treatment monitoring and prognosis of tumors in dogs and decide which companion animals should be treated.

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Appendix:

1) Amino acid composition of:

   a) Dog cytosolic TK1

   b) Dog TK1 (wild type)

   c) Recombinant TK1 C-12

   d) Cytoslic Human TK1

   e) Recombinant human TK1

2) Peptide analysis results.

3) Dog and Human TK1 amino acid sequence.

a) Predicted amino acid composition of full length cytosolic TK1 from the dog gene sequenced: (Wang L et al in preparation) original sequence is from Gene bank.

Predicted Structural Class of the Whole Protein: Alpha
Deléage & Roux Modification of Nishikawa & Ooi 1987

Analysis	Whole Protein
Molecular Weight	26449.83 m.w.
Length	242
1 microgram =	37.807 pMoles
Molar Extinction coefficient	8640±5%
1 A(280) =	3.06 mg/ml
Isoelectric Point	8.73
Charge at pH 7	7.19

Whole Protein Composition Analysis

Amino Acid(s)	Number count	% by weight	% by frequency
Charged (RKHYCDE)	72	36.17	29.75
Acidic (DE)	24	11.29	9.92
Basic (KR)	31	16.50	12.81
Polar (NCQSTY)	59	24.54	24.38
Hydrophobic (AILFWV)	86	33.70	35.54
A Ala	21	5.64	8.68
C Cys	8	3.12	3.31
D Asp	8	3.48	3.31
E Glu	16	7.81	6.61
F Phe	11	6.12	4.55
G Gly	19	4.10	7.85
H His	3	1.56	1.24
I Ile	11	4.71	4.55
K Lys	17	8.24	7.02
L Leu	21	8.98	8.68
M Met	7	3.47	2.89
N Asn	8	3.45	3.31
P Pro	13	4.77	5.37
Q Gln	9	4.36	3.72
R Arg	14	8.27	5.79
S Ser	15	4.94	6.20
T Thr	13	4.97	5.37
V Val	22	8.25	9.09
W Trp	0	0.00	0.00
Y Tyr	6	3.70	2.48
B Asx	0	0.00	0.00
Z Glx	0	0.00	0.00
X Xxx	0	0.00	0.00
. Ter	1	0.00	0.41

b) Predicted amino acid composition of recombinant full length TK1 from the dog (Wang L et al in preparation) original sequence is from Gene bank.

Predicted Structural Class of the Whole Protein: Alpha
Deléage & Roux Modification of Nishikawa & Ooi 1987

Analysis	Whole Protein
Molecular Weight	29192.73 m.w.
Length	266
1 microgram =	34.255 pMoles
Molar Extinction coefficient	9920±5%
1 A(280) =	2.94 mg/ml
Isoelectric Point	8.59
Charge at pH 7	7.52

Whole Protein Composition Analysis

Amino Acid(s)	Number count	% by weight	% by frequency
Charged (RKHYCDE)	82	37.53	30.83
Acidic (DE)	25	10.67	9.40
Basic (KR)	31	14.95	11.65
Polar (NCQSTY)	67	25.11	25.19
Hydrophobic (AILFWV)	89	31.81	33.46
A Ala	21	5.11	7.89
C Cys	8	2.83	3.01
D Asp	8	3.15	3.01
E Glu	17	7.52	6.39
F Phe	12	6.05	4.51
G Gly	22	4.30	8.27
H His	11	5.17	4.14
I Ile	11	4.26	4.14
K Lys	17	7.46	6.39
L Leu	23	8.92	8.65
M Met	8	3.60	3.01
N Asn	9	3.52	3.38
P Pro	13	4.32	4.89
Q Gln	10	4.39	3.76
R Arg	14	7.49	5.26
S Ser	20	5.97	7.52
T Thr	13	4.50	4.89
V Val	22	7.47	8.27
W Trp	0	0.00	0.00
Y Tyr	7	3.91	2.63
B Asx	0	0.00	0.00
Z Glx	0	0.00	0.00
X Xxx	0	0.00	0.00
. Ter	1	0.00	0.38

c) Predicted amino acid composition of recombinant TK1 of dog with His tag protein Δ 12 (lacking the 12 C-terminal amino acids) (Wang L et al in preparation). Original sequence is from Gene bank.

Predicted Structural Class of the Whole Protein: Alpha
Deléage & Roux Modification of Nishikawa & Ooi 1987

Analysis	Whole Protein
Molecular Weight	27946.31 m.w.
Length	254
1 microgram =	35.783 pMoles
Molar Extinction coefficient	9800±5%
1 A(280) =	2.85 mg/ml
Isoelectric Point	8.67
Charge at pH 7	7.38

Whole Protein Composition Analysis

Amino Acid(s)	Number count	% by weight	% by frequency
Charged (RKHYCDE)	80	38.35	31.50
Acidic (DE)	25	11.15	9.84
Basic (KR)	31	15.62	12.20
Polar (NCQSTY)	62	24.23	24.41
Hydrophobic (AILFWV)	85	31.96	33.46
A Ala	19	4.83	7.48
C Cys	7	2.58	2.76
D Asp	8	3.29	3.15
E Glu	17	7.85	6.69
F Phe	12	6.32	4.72
G Gly	22	4.49	8.66
H His	10	4.91	3.94
I Ile	11	4.45	4.33
K Lys	17	7.80	6.69
L Leu	22	8.91	8.66
M Met	8	3.76	3.15
N Asn	8	3.27	3.15
P Pro	11	3.82	4.33
Q Gln	8	3.67	3.15
R Arg	14	7.82	5.51
S Ser	19	5.92	7.48
T Thr	13	4.70	5.12
V Val	21	7.45	8.27
W Trp	0	0.00	0.00
Y Tyr	7	4.09	2.76
B Asx	0	0.00	0.00
Z Glx	0	0.00	0.00
X Xxx	0	0.00	0.00
. Ter	0	0.00	0.00

d) Predicted amino acid composition of full length human recombinant TK1 with His tag protein (Wang L et al in preparation) original sequence is from Gene bank.

Predicted Structural Class of the Whole Protein: Alpha
Deléage & Roux Modification of Nishikawa & Ooi 1987

Analysis	Whole Protein
Molecular Weight	28242.74 m.w.
Length	258
1 microgram =	35.407 pMoles
Molar Extinction coefficient	10280±5%
1 A(280) =	2.75 mg/ml
Isoelectric Point	8.40
Charge at pH 7	7.26

Whole Protein Composition Analysis

Amino Acid(s)	Number count	% by weight	% by frequency
Charged (RKHYCDE)	80	37.49	31.01
Acidic (DE)	23	10.12	8.91
Basic (KR)	29	14.45	11.24
Polar (NCQSTY)	66	25.92	25.58
Hydrophobic (AILFWV)	87	31.68	33.72
A Ala	25	6.29	9.69
C Cys	11	4.02	4.26
D Asp	8	3.26	3.10
E Glu	15	6.86	5.81
F Phe	12	6.25	4.65
G Gly	20	4.04	7.75
H His	10	4.86	3.88
I Ile	12	4.81	4.65
K Lys	16	7.26	6.20
L Leu	20	8.01	7.75
M Met	8	3.72	3.10
N Asn	8	3.23	3.10
P Pro	15	5.16	5.81
Q Gln	12	5.44	4.65
R Arg	13	7.19	5.04
S Ser	17	5.24	6.59
T Thr	11	3.94	4.26
V Val	18	6.32	6.98
W Trp	0	0.00	0.00
Y Tyr	7	4.04	2.71
B Asx	0	0.00	0.00
Z Glx	0	0.00	0.00
X Xxx	0	0.00	0.00
. Ter	1	0.00	0.39

e) Predicted amino acid composition of full length cytosolic TK1 from human gene sequenced: (Wang L et al in preparation) original sequence is from Gene bank.

Predicted Structural Class of the Whole Protein: Alpha
Deléage & Roux Modification of Nishikawa & Ooi 1987

Analysis	Whole Protein
Molecular Weight	25499.84 m.w.
Length	234
1 microgram =	39.216 pMoles
Molar Extinction coefficient	9000±5%
1 A(280) =	2.83 mg/ml
Isoelectric Point	8.51
Charge at pH 7	6.92

Whole Protein Composition Analysis

Amino Acid(s)	Number count	% by weight	% by frequency
Charged (RKHYCDE)	70	36.07	29.91
Acidic (DE)	22	10.70	9.40
Basic (KR)	29	16.00	12.39
Polar (NCQSTY)	58	25.41	24.79
Hydrophobic (AILFWV)	84	33.63	35.90
A Ala	25	6.97	10.68
C Cys	11	4.45	4.70
D Asp	8	3.61	3.42
E Glu	14	7.09	5.98
F Phe	11	6.35	4.70
G Gly	17	3.80	7.26
H His	2	1.08	0.85
I Ile	12	5.33	5.13
K Lys	16	8.04	6.84
L Leu	18	7.99	7.69
M Met	7	3.60	2.99
N Asn	7	3.13	2.99
P Pro	15	5.71	6.41
Q Gln	11	5.53	4.70
R Arg	13	7.96	5.56
S Ser	12	4.10	5.13
T Thr	11	4.36	4.70
V Val	18	7.00	7.69
W Trp	0	0.00	0.00
Y Tyr	6	3.84	2.56
B Asx	0	0.00	0.00
Z Glx	0	0.00	0.00
X Xxx	0	0.00	0.00
. Ter	1	0.00	0.43

2) Peptide analysis of 60-70 KDa band of∆ 12 Recombinant dog TK1:

1. gi|73964971 **Mass:** 26890 **Score:** 110 **Expect:** 9.8e-05 **Queries matched:** 9
PREDICTED: similar to Thymidine kinase, cytosolic [Canis familiaris]

Observed	Mr(expt)	Mr(calc)	Delta	Start	End	Miss	Peptide
1053.5846	1052.5774	1052.5767	0.0007	42 -	49	1	R.RFQIAQYK.C
1213.5344	1212.5271	1212.5160	0.0111	61 -	70	0	R.YSNSFSTHDR.N
1286.5804	1285.5731	1285.5617	0.0114	149 -	158	0	K.LTAVCMECFR.E
1302.5702	1301.5629	1301.5567	0.0062	149 -	158	0	K.LTAVCMECFR.E + Oxidation (M)
1388.6976	1387.6903	1387.6952	-0.0049	71 -	82	0	R.NTMEALPACLLR.D
1390.7787	1389.7714	1389.7616	0.0098	118 -	130	0	K.TVIVAALDGTFQR.K
1490.7945	1489.7873	1489.7963	-0.0090	19 -	32	0	R.GQIQVILGPMFSGK.S + Oxidation (M)
1818.8771	1817.8698	1817.8730	-0.0032	171 -	186	1	K.EVEVIGGADKYHSVCR.L
1907.0348	1906.0275	1906.0524	-0.0249	204 -	221	1	R.ENKENVLVLVPGKPGEGK.E

No match to: 825.0922, 850.0390, 861.0595, 877.0331, 1147.5657, 1227.6845, 1309.6606, 1360.6919, 1374.7155, 1567.7700, 1652.8611, 1665.9137, 1751.8219, 1848.8621, 2105.1012

Match to: **gi|73964971** Score: **110** Expect: **9.8e-05**
PREDICTED: similar to Thymidine kinase, cytosolic [Canis familiaris]
Nominal mass (M_r): **26890;** Calculated pI value: **8.99**

(Source: Å. Engstrom et al .., IMBIM, Uppsala University)

Dog and Human TK1 amino acid sequence

	10	20	30	40	50	60
Dog	MSCINLPTVL	PGSPSKTRGQ	IQVILGPMFS	GKSTELMRRV	RRFQIAQYKC	LVIKYAKDTR
Human	MSCINLPTVL	PGSPSKTRGQ	IQVILGPMFS	GKSTELMRRV	RRFQIAQYKC	LVIKYAKDTR

	70	80	90	100	110	120
Dog	YSNSFSTHDR	VAVIGIDEGQ	NTMEALPACL	LRDVAQEALG	FFPDIVEFSE	TMANAGKTVI
Human	YSSSFCTHDR	NTMEALPACL	LRDVAQEALG	VAVIGIDEGQ	FFPDIVEFCE	AMANAGKTVI

	130	140	150	160	170	180
Dog	VAALDGTFQR	KAFGTILNLV	PLAESVVKLT	AVCMECFREA	AYTKRLGSEK	EVEVIGGADK
Human	VAALDGTFQR	KPFGAILNLV	PLAESVVKLT	AVCMECFREA	AYTKRLGTEK	EVEVIGGADK

	190	200	210	220	230
Dog	YHSVCRLCYF	KKASGPPMGL	DSERNKENVL	VLVPGKPGEG	KEATGVRKLF
Human	YHSVCRLCYF	KKASGQPAGP	DNKEN.....	CPVPGKPGE.	..AVAARKLF

	240
Dog	APQHVLQCSPAN
Human	APQQILQCSPAN

- Red color indicates the difference in the amino acid sequence.