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Research paper

Loss of CD45 cell surface expression in canine T-zone lymphoma results from reduced gene expression

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ABSTRACT

Canine T-zone lymphoma (TZL) is a peculiar lymphoma subtype characterized by an indolent clinical course and aberrant CD45-negative phenotype, commonly recognized by flow cytometry (FC). Recent studies have described clinical presentation and behavior, but to date the mechanisms behind the loss of CD45 protein expression have never been investigated.

The aims of this study were: 1) to confirm the absence of CD45 in canine TZL via the concomitant use of FC and immunohistochemistry with two different sources of antibody; and 2) to investigate the amount of CD45 transcript and the presence of CD45 gene in the neoplastic cells of dogs affected by TZL.

57 lymph node aspirates were included in the present study: 40 (70.2%) TZLs, 7 (12.3%) high grade T-cell lymphomas and 10 (17.5%) reactive lymph nodes. Neoplastic cells and normal T-cells were isolated from TZL and reactive lymph nodes, respectively, via cell sorting. Immunohistochemistry was performed on 2 TZL, 2 reactive lymph nodes and 2 Peripheral T-cell Lymphomas. Total RNA and genomic DNA were extracted from lymph-nodes aspirates. Two different quantitative real-time PCR experiments were designed, to determine the amount of the CD45 transcript and of the corresponding gene fragment.

All TZL cases were negative for CD45 at immunohistochemistry. CD45 transcript amount was significantly lower in TZL compared to controls ($p < 0.001$). This difference was not significant ($p = 0.584$) for CD45 DNA load, that was similar between TZL and controls. Moreover, CD45 transcript amount was inversely correlated with the percentage of neoplastic cells in each TZL sample ($p = 0.010$).

These results confirm that CD45 protein is lacking on cell surface irrespective of the technique and antibody source adopted. This phenotypic aberrancy is apparently due to the absence of gene transcription, as CD45 DNA was present, whereas CD45 transcript was virtually absent in the neoplastic cells. The data here reported support further studies investigating possible factors impairing CD45 gene transcription.

1. Introduction

Dogs are frequently affected by hematopoietic malignancies, with lymphoma being the most represented. Nevertheless, many different entities are grouped together under the diagnosis of lymphoma, showing different clinical presentation and outcome (Valli et al., 2013).

Small clear cell/T-zone lymphoma (TZL) subtype has been recently identified as a unique entity with a peculiar disease distribution at onset and with a typical indolent clinical course (Seelig et al., 2014; Martini et al., 2015a). Histologically, TZL shows neoplastic cells expanding the paracortex and medullary cords without effacing the nodal architecture; neoplastic cells are small lymphocytes with sharp, shallow nuclear indentations, unapparent nucleoli and a moderate volume of pale cytoplasm. Mitoses are rare (Valli et al., 2006). Cytological aspirates in dogs diagnosed with TZL are characterized by a prevalent population of small lymphoid cells with a clear cytoplasm and frequent hand mirror shape ("small clear cells" appearance). The mitotic index is low (Fournel-Fleury et al., 2002). Finally, a peculiar phenotype has been identified by flow cytometry (FC), characterized by the expression of a variable number of T-cell markers and loss of expression of CD45 (Martini et al., 2015b; Seelig et al., 2014).

CD45 is a transmembrane protein tyrosine phosphatase expressed

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by the leukocytes surface in many different species, including dog (Goto-Koshino et al., 2014). In humans, alternative RNA splicing causes the generation of various CD45 isoforms, differing for molecular weight and biological activity (Dupéré-Minier et al., 2010). Accordingly, two major CD45 isoforms (CD45RA and CD45R0) have been described in dog and expression patterns are different depending on leukocytes subclasses and activation status (Goto-Koshino et al., 2014).

Historically, most of the studies have been centred to explain the role of CD45 either in cell maturation or in signal transduction of T- and B-cells after antigenic stimulation (Pingel and Thomas, 1989; Kishihara et al., 1993; Byth et al., 1996), whereas more recently CD45 has been proposed as apoptosis regulator (Lesage et al., 1997; Fortin et al., 2002; Pang et al., 2009).

CD45-negative hematopoietic neoplasms other than canine TZL have been reported both in humans (Ozdemirli et al., 1996; Ratei et al., 1998; Kumar et al., 2005; Wang et al., 2015) and dogs (Gelain et al., 2008; Williams et al., 2008), but the mechanisms underlying this phenotypic aberrancy have not been investigated. To test this, the present study has been organized in different tasks. The first aim is to confirm the absence of CD45 at the protein level in a group of dogs with TZL via the concomitant use of FC and immunohistochemistry with two different sources of antibody. Based on of the absence of CD45 protein in cTZL, subsequently the amount of CD45 transcript and the presence of CD45 gene will be investigated in the same cohort of dogs. For the purpose of the study a control group composed by normal sorted T-cells, high grade T-cell lymphomas and normal lymph nodes has been selected. This stepwise approach will outline the possible origin of CD45 protein loss, whether associated to genetic or transcriptional or post-translational modifications.

2. Materials and methods

2.1. Case selection

Samples from the present study were collected from the flow cytometric diagnostic services of Department of Veterinary Medicine (University of Milan, Milan, Italy) and Department of Veterinary Sciences (University of Turin, Grugliasco, Turin, Italy). Consecutive cases with a final diagnosis of TZL based on cytological appearance and FC immunophenotype (Martini et al., 2015b) were included. In addition, cases of non-neoplastic lymph node and of high grade T-cell lymphoma showing CD45 expression were included as controls. When available, histopathological sections of the same cases were considered to confirm the diagnosis and perform immunohistochemical analyses.

All dogs were privately owned and sampled for diagnostic purposes with a written informed consent of the owners. Thus, a formal approval of the Institution Committee for Animal Care was not required.

2.2. Flow cytometry

Lymph node aspirates collected into 1 ml RPMI tubes (Sigma Aldrich, St. Louis, MO, USA) were processed for FC as already described (Gelain et al., 2008), using a wide antibody panel (Table 1). All antibodies had been previously titred to choose the optimal working dilutions. A multicolour approach was used, allowing the assessment of the contemporary expression of different antigens in the same cellular population (Martini et al., 2015b). If necessary, RBCs were lysed by means of a buffer containing 8% ammonium chloride after antibody incubation.

Samples were acquired either with a FACScalibur or a BD Accuri C6 (Becton Dickinson, San Josè, CA, USA) and analysed using the specific software CellQuest or CFlow Plus (Becton Dickinson).

The percentage of CD45-negative neoplastic cells was obtained in all the TZLs.

Table 1
antibody panel used to label lymph node aspirates collected into RPMI tubes from dogs with suspected lymphoma.

Target molecule	Antibody clone	Source	Specificity
CD45	YKIX716.13	Serotec, Oxford, UK	All leukocytes
CD3	CA17.2A12	Serotec	T-cells
CD5	YKIX322.3	Serotec	T-cells and a subset of B-cells
CD4	YKIX302.9	Serotec	T-helper cells and neutrophils
CD8	YCATE55.9	Serotec	T-cytotoxic cells
CD21	CA2.1D6	Serotec	Mature B-cells
CD34	1H6	BD Pharmingen	Precursors

2.3. Cell sorting

Neoplastic cells (CD45-negative and CD5-positive) from a subset of TZLs and normal T-cells (CD45-positive and CD5-positive) from non-neoplastic lymph nodes were sorted using a fluorescence-activated cell sorter (BD FACSVantage, BD Biosciences, San Josè, CA, USA). A minimum sorting yield of 1×10^5 with more than 95% purity was considered suitable for quantitative real-time PCR analysis.

2.4. Histopathology and immunohistochemistry

For histological examination, three μm sections were stained with haematoxylin and eosin (HE).

For lymphoma phenotyping, a panel of antibodies was applied including: monoclonal mouse anti-human CD3 (Clone F7.2.38, Dako, Atlanta, Georgia, USA, T cells; diluted 1:100), monoclonal mouse antihuman CD5 (Clone CD5/54/F6, Dako, T cells; diluted 1:100), monoclonal mouse anti-human CD79acy (Clone HM57, Dako, all stages of B cells; diluted 1:100) and CD20 epitope-specific rabbit antibody (RB-9013-P, Thermo Fisher Scientific Inc., Cheshire, UK; mature B cells; diluted 1:800). The primary antibody incubation step was performed by an automated system for all antibodies (Ventana Medical Systems, Tucson, Arizona, USA).

Immunohistochemical analysis for CD45 was performed using H2O2 3% in methanol for 30 min at room temperature for blocking endogenous peroxidase activity. Sections underwent high temperature antigen unmasking by incubation with citric acid buffer (pH 6) at 98 °C. The antibody used was an anti-canine CD45, clone CA12.10C12 (Leukocyte Antigen Biology Laboratory, UCDavis, Davis, CA, USA) (dilution 1:100). Antibody was detected using an avidine-biotine-peroxidase complex technique with the Vectastain Elite ABC Kit (Vector Laboratories Inc., Burlingame, California, USA). For negative controls, the primary antibody was excluded during the process. Membranous immunolabelling of the antibodies was evaluated in neoplastic cells in lymphomas and in the control lymph nodes. Evaluation consisted of qualitative assessment of cell types and location.

2.5. Quantitative real-time RT-PCR for CD45 transcript

The transcript analysis was performed on lymph node aspirates diluted in RNAlater® (Applied Biosystems, Life Technologies, Carlsbad, CA) and stored at −20 °C until processing. The total RNA was isolated from cell pellets using the RNeasy Mini Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. To avoid genomic DNA contamination, on-column DNase digestion with the RNase-Free DNase (Qiagen) set was performed. Total RNA concentration and quality were measured with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). First-strand cDNA was synthesised from 100 ng of total RNA using High Capacity cDNA Reverse Transcription Kit (Invitrogen, Life Technologies, Carlsbad, CA) accord-

ing to the manufacturer's protocol. The generated cDNA was used as template for quantitative real-time RT-PCR (qRT-PCR) in a LightCycler 480 Instrument (Roche Diagnostics, Basel, Switzerland) using standard PCR conditions. The qRT-PCR reactions consisted of 5 µl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Life Technologies, Carlsbad, CA), 0.3 µl of forward and reverse primers (10 µM) (the primer combination and final concentrations were optimized during assay setup) and 2.5 µl of diluted (1–40) cDNA. The primers were designed using Primer Express 2.0 (Applied Biosystem, Life Technologies, Carlsbad, CA). Canine CD45 was amplified using the primer pair 5′- ATG GAG ATG CAG GGT CAA AT-3′ (forward) and 5′-GCA ATG TAT TTC CTG GGT TCT T-3′ (reverse). Primer pairs were designed on exon 19 and 20, shared by the two CD45 variants. In addition, these exons spare an intronic region of > 3800 bp thus excluding the amplification of contaminant genomic DNA. Calibration curves using a 7-fold serial dilution (1:2) of a cDNA pool revealed PCR efficiencies of 99.5%. Canine transmembrane BAX inhibitor motif containing 4 (CGI-119) was chosen as reference gene for the absence of pathological state dependent differences in mRNA expression, as reported by Aricò et al. (2013). ΔΔCt method (Livak and Schmittgen, 2001) was used for the relative quantification of mRNA, ultimately expressed as Relative Expression (RE).

2.6. Quantitative real-time PCR for CD45 DNA

An aliquot of lymph node aspirates was stored at −20 °C until processing for DNA load analysis. Genomic DNA was extracted from cell pellets using DNeasy® Blood & Tissue kit (Qiagen) following the manufacturer's instructions. Total DNA concentration and quality were measured with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies). Primers were designed using Primer3web (primer3.ut.ee) based on the corresponding canine genome project gene sequences available through Ensembl web site (www.ensembl.org). Specific primers for CD45 were designed as follows: primer pair CD45 5′-AGCAAAGACACACGAAAGCC-3′ (forward) and 5′-GCAATGTATTT CCTGGGTTCT −3′ (reverse) for the amplification of a fragment of 257 base pairs. In order to detect reference genes for normalization of samples, primers targeting canine CGI-119 and canine GAPDH was designed as described: primer pair CGI-119 5′-GGATTTTGTG CTTGTCAGGAA-3′ (forward) and 5′-CACTGGGAGCTTAGCAATTACA-3′ (reverse) for the amplification of a fragment of 279 base pairs; primer pairs GAPDH 5′-GGAGAAAGCTGCCAAATATG-3′ (forward) and 5′-ACCAGGAAATGAGCTTGACA-3′ (reverse) for the amplification of a fragment of 194 base pairs (Mortarino et al., 2009). Quantitative Real-Time PCR was performed in 14 µl with 1 µl of target DNA, using 1x Eva Green mix (Biorad, Segrate, Milan, Italy), and 10 µM of each primer. Samples were tested in duplicate. In order to evaluate the PCR efficiency using a relative standard curve, dilution series were prepared by performing four fold serial dilution (1:10) of a control sample. Efficiency was 100.47% for CD45, 102.16% for CGI119 and 98.70 for GAPDH. ΔΔCt method (Livak and Schmittgen, 2001) was used to describe the CD45 DNA load to the reference genes, expressed as Relative Quantification (RQ).

2.7. Amplicon sequencing

The specificity of the amplification for CD45 transcript and DNA was checked by amplicon DNA sequencing. Briefly, the reaction mixtures after PCR were run by 2.5% agarose gel electrophoresis, and the UV-visualized bands were purified using the QiaquickTM Gel Extraction Kit (Qiagen GmbH, Hilden, Germany). The concentration of the purified amplicons was assessed using a ND-100 spectrophotometer (NanoDrop, Wilmington, DE USA). Then, each amplicon was sequenced by Sanger chemistry (Eurofins Genomics S.r.l., Vimodrone, MI, Italy). The obtained sequences were aligned with the expected target sequence using BLAST (https://blast.ncbi.nlm.nih.gov).

2.8. Statistical analysis

Data distribution was assessed via Shapiro-Wilk test for each subset.

Kruskal-Wallis test was used to compare CD45 transcript amount among TZL, cells CD5-positive sorted from reactive lymph nodes and CD45-positive high grade T-cell lymphomas. A Mann-Whitney test was used to compare CD45 transcript amount between TZL samples with < 95% and > 95% neoplastic cells.

The Kruskal-Wallis test was also performed to compare CD45 DNA load among TZL, reactive lymph nodes and cells CD45-negative and CD5-positive sorted from TZL.

A non-parametric Spearman correlation analysis was used to determine the potential relationship between CD45 transcript amount and percentage of neoplastic cells in the TZL samples.

All analyses were performed with standard statistical software (SPSS v20.0 for Windows) and significance was set at $p \leq 0.05$ for all tests.

3. Results

Fine needle aspirates from an enlarged lymph node were obtained from 57 dogs and included in the study. Diagnoses were as follow: 40 (70.2%) TZLs, 7 (12.3%) high-grade T-cell lymphomas and 10 (17.5%) hyperplastic lymphadenopathy. Normal and neoplastic cells were obtained with cell sorting from 3 non-neoplastic lymph nodes and 3 TZLs, respectively. In addition, paraffin embedded lymph nodes from 2 cases diagnosed as reactive hyperplasia, 2 as Peripheral T-cell lymphomas (PTCL) and 2 as TZL were available for immunohistochemical analysis.

According to FC analysis, the percentage of CD45-negative neoplastic cells in the TZLs was 80.54 ± 14.9% (median 82.6%; min-max 30.77–98.04%). The composition of the residual CD45-positive population was heterogeneous. One sample had a predominant (94.5%) population of granulocytes and the lymphoid population was composed of T-cytotoxic (4.8%), T-helper (1.4%) and B-cells (0.34%): this sample was highly hemo-contaminated at cytological examination. For the remaining samples, the residual CD45-positive population was composed of lymphoid cells alone, with a mean percentage of 36.57 ± 17.31% (median 33.22%; min-max 1.40–67.41%) of B-cells, 34.72 ± 19.45% (median 33.63%; min-max 1.89–92.09%) of T-helper cells, and 18.75 ± 14.64% (median 12.91%; min-max 0.72–60.15%) of T-cytotoxic cells. As expected, the CD45-negative and CD5-positive cells were not detected either in the reactive lymph nodes or in the high-grade T-cell lymphomas.

Immunohistochemical results showed a diffuse CD45 membranous expression in PTCLs. In the reactive lymph nodes, CD45 positive cells were located both in the paracortex and in the germinal centres (Fig. 1); cells in the marginal and mantle zones were also CD45 positive, even with a lower intensity. TZLs were diffusely negative (Fig. 2).

Amplicon DNA sequencing confirmed (99–100%) the specificity of the amplification for both CD45 targets.

Data concerning CD45 transcript amount were obtained from 41 cases, including 31 (75.6%) TZLs, 7 (17.1%) high grade T-cell lymphomas, and 3 (7.3%) sorted normal T cells. The mean neoplastic cells percentage in TZLs undergoing transcript analysis was 80.23 ± 15.61% (median 82.6%; min-max 30.77–98.04%); in particular, 26 (83.9%) cases had < 95% neoplastic cells, whereas the remaining 5 (16.1%) cases had > 95% neoplastic cells. Results of transcript analysis are shown in Fig. 3.

A significant difference in the CD45 transcript amount was detected among TZLs, normal T cells and high grade T-cell lymphomas ($p < 0.001$). mRNA levels were significantly lower in TZLs than in normal T cells and high grade T-cell lymphomas ($p < 0.001$), respectively. No significant difference was found between normal T cells and high grade T-cell lymphomas ($P = 0.138$).

When exploring the correlation between the amount of CD45 transcript and the percentage of the neoplastic cells in TZL, a significant

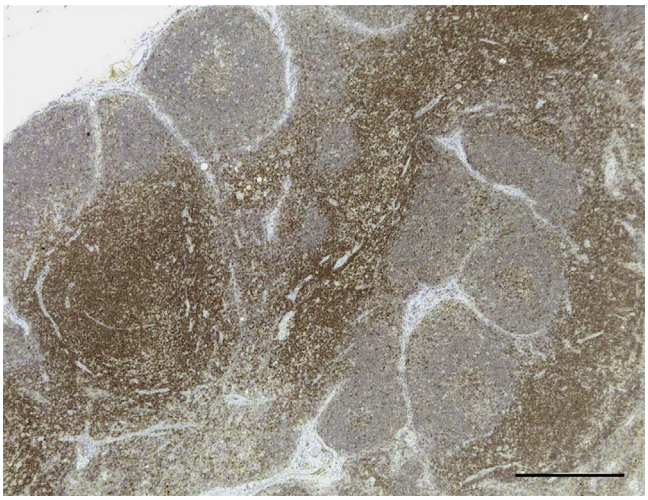

Fig. 1. Representative image of CD45 protein staining in a reactive lymphoid hyperplasia.T-lymphocytes within the paracortex and B-lymphocytes in the follicles show a diffuse CD45 immunolabelling. Bar: 500 μm.

inverse correlation was detected between CD45 mRNA data and the percentage of neoplastic cells ($p = 0.010$). In addition, the amount of CD45 transcript was higher in the 26 TZL samples with < 95% neoplastic cells (mean = 0.37 ± 0.29) than in the 5 TZL samples with > 95% neoplastic cells (mean = 0.23 ± 0.16), although the difference was not statistically significant.

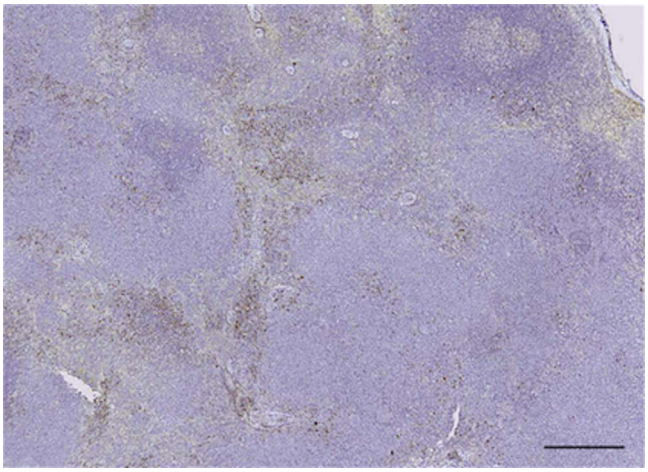

Fig. 2. Representative image of CD45 protein staining in a T-zone lymphoma.Neoplastic lymphocytes are negative to CD45 immunolabelling. Bar: 500 μm.

Data concerning CD45 DNA load were obtained for 33 samples, including 24 (72.7%) TZLs, 3 (9.1%) CD45-negative cells and CD5-positive cells sorted from TZLs and 6 (18.2%) non-neoplastic lymph nodes. The mean neoplastic cells percentage in TZLs undergoing DNA analysis was 81.95 ± 9.88% (median 83.26%; min-max 30.77–96.5%). Results are shown in Fig. 3. The differences among subsets were not significant irrespective of the reference gene considered ($p = 0.165$ for CGI-119 and $p = 0.895$ for GAPDH).

4. Discussion

The absence of CD45 expression in canine TZL has been previously described to demonstrate the diagnostic efficiency and the staging prediction (Martini et al., 2015a,b; Seelig et al., 2014). Interestingly, all these studies assessed the expression of the protein by FC using the same antibody clone (YKIX716.13), although conjugated with different fluorochromes. As a consequence, protein modifications preventing antibody binding during FC processing could not be completely excluded based on the published literature. In the present study, we analysed two cases of TZL via immunohistochemistry using a different antibody clone and results confirmed FC analysis. Both techniques and clones could not detect CD45 protein, thus confirming the lack of CD45 protein expression in canine TZL. Although no specific information was available about the sequence recognized by the two different antibodies, these preliminary results suggested that partial modification of the protein is unlikely and supported the hypothesis of a complete loss of CD45 in TZLs.

To investigate this scenario, we inquired whether CD45 transcript was also reduced in canine TZL and consequently if CD45 gene was still represented in the genome of the neoplastic cells. To answer this hypothesis, we designed two quantitative real-time PCR experiments in order to investigate the transcript amount and DNA load, respectively.

In the study setting a major limiting factor was related to the selection of control lymphocytes for both transcript and DNA analyses. Indeed, CD45 is differently expressed in the various canine leukocyte subclasses, also depending on the activation status (Goto-Koshino et al., 2014), and CD45 transcript amount may vary accordingly. In particular, CD45 is expressed at lower levels in B-cells than in T-cells (Comazzi et al., 2006): thus, inclusion of B-cells in the control group for transcript amount analysis would have affected results concealing possible differences between TZLs and controls. Respect to this, we selected as control high grade T-cell lymphomas and a pure T-cell population obtained by cell sorting from non-neoplastic lymph nodes.

Based on our results, CD45 transcript amount resulted significantly lower in TZLs compared to high-grade T-cell lymphomas and normal T-cells. Albeit a small CD45 transcript amount was detected in TZLs, this might be associated to the contamination of relevant residual normal lymphocytes in the samples obtained. This was well demonstrated when

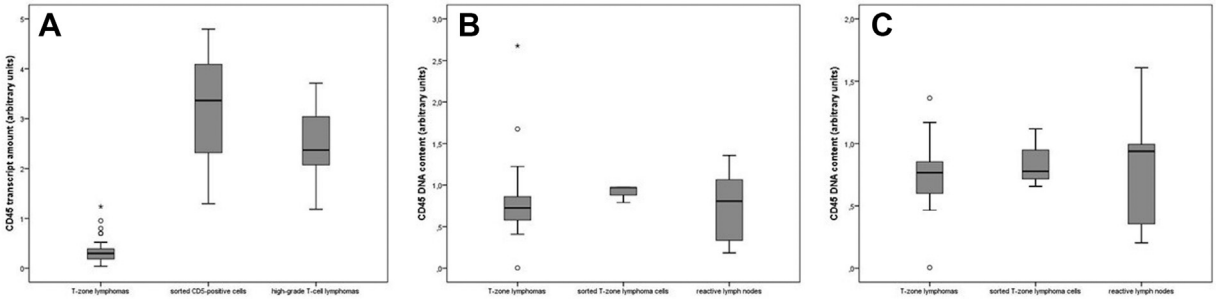

Fig. 3. CD45 transcript and DNA amount in canine T-zone lymphomas and in controls.Panel A: CD45 transcript amount in canine T-zone lymphomas, sorted CD5-positive cells from reactive lymph nodes and high-grade T-cell lymphomas; CGI-119 was used as a housekeeping gene; CD45 transcript amount was significantly lower in T-zone lymphomas than in the other two groups, respectively ($p < 0.001$). Panel B: CD45 DNA load in canine T-zone lymphomas, sorted neoplastic cells from T-zone lymphomas and reactive lymph nodes; CGI-119 was used as a reference gene; no significant difference in CD45 DNA load was found among groups. Panel C: CD45 DNA load in canine T-zone lymphomas, sorted neoplastic cells from T-zone lymphomas and reactive lymph nodes; GAPDH was used as a reference gene; no significant difference in CD45 DNA load was found among groups.

comparing the CD45 gene expression to the number of neoplastic cells in these tumors. The lowest CD45 transcript amounts were associated to TZLs with the highest percentage of neoplastic cells and consequently the lowest percentage of residual lymphocytes. Taken together, these results suggest that CD45 transcript is virtually absent in TZL neoplastic cells.

For DNA load analysis, high grade T-cell lymphomas were excluded as controls due to the possible presence of genetic abnormalities that occur in cancer cells, leading to unpredictable biases. In contrast, the selection of non-neoplastic lymph nodes as controls, potentially should not interfere with this result, as variations in genes copy number are not expected in reactive lymph nodes, at least in the CD45 gene. However, the silico identification of the reference gene for DNA load analysis was complicated by two biological variables. First, copy number variations may eventually occur at gene level biasing the results and second, no relevant data are published about genetic modifications of canine TZL. Thus, primers for two different genes were designed for our analysis and results showed a similar load in TZLs and controls, irrespective of the reference gene used, demonstrating that CD45 gene is not deleted in canine TZL.

Primers design was also challenging, as different isoforms of CD45 exist obtained by alternative RNA splicing (Dupéré-Minier et al., 2010) and the gene comprises many introns (www.ensembl.org). Furthermore, we designed primers that were adapted to amplify a transcript fragment common to all CD45 isoforms, and a gene segment specifically encoding for the transcript fragment was amplified. By doing this, we reached a double goal: first, we included all CD45 isoforms in our analysis; second, we described the different fate of a single sequence at two different levels (transcript and DNA).

Many different mechanisms may cause CD45 gene transcription switch off, including genetic, epigenetic, and genomic abnormalities. Still, gross chromosome or gene deletions are unlikely. Indeed, we were able to detect the presence of CD45 gene in TZL cells. In addition, Seelig et al. (2014) report that there is no evident loss of the telomeric end of chromosome 7, where CD45 is located, although no scientific data are described in their publication. All other mechanisms are still possible and should be evaluated via further studies.

Different studies in human medicine investigated the importance of CD45 in normal T- and B-cells, highlighting its role in cell maturation, signal transduction and apoptosis mediator (Pingel and Thomas, 1989; Kishihara et al., 1993; Byth et al., 1996; Lesage et al., 1997; Fortin et al., 2002; Pang et al., 2009). Its involvement in neoplastic transformation may be suspected, but this has never been demonstrated. Interestingly, different oncogenes are located near CD45 gene in canine chromosome 7, including ABL2, AKT3, YES1 and some members of the Ras family (www.ensembl.org). These oncogenes play a role in cancer development in human leukemias and lymphomas (Mao et al., 2003; Huang et al., 2010; Kim et al., 2015; Roberts and Mullighan, 2015). Perturbation of methylation on the promoter regions of these genes or genomic abnormalities on a large segment of canine chromosome 7 may cause impaired transcription of any of these oncogenes participating to the tumorigenesis. Thus, it cannot be excluded that the absence of CD45 protein is only a phenotypic epiphenomenon, with minimal involvement in the tumor biology. Further studies are needed, assessing possible alterations in oncogenes activation.

The whole CD45 gene sequencing would probably solve the limitations of our study where only a fragment of CD45 gene specifically encoding for the transcript fragment selected was amplified. Thus, mutations, short deletions or base pair variations may occur upstream of the amplified fragment, remaining unnoticed to our analysis, but still preventing gene transcription.

In conclusion, the present study confirms the lack of CD45 protein in canine TZL, irrespective of the different techniques and antibody clones used. Based on our results, this phenotypic aberrancy is likely due to the absence of gene transcription, as CD45 DNA was present, whereas CD45 transcript was virtually absent in the neoplastic cells. Further studies are needed to identify the abnormalities causing the lack of transcription.

Conflict of interest

We disclose any possible conflict of interest.

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