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Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/149923> since 2018-01-21T11:04:35Z

Published version:

DOI:10.1016/j.vetimm.2014.02.008

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CD44 in canine leukemia: Analysis of mRNA and protein expression in peripheral blood

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Abstract

Hyaluronan receptor CD44 mediates interaction between cells and extracellular matrix. The expression of standard form and its variants is dysregulated in human leukemias and is associated with metastasis and prognosis. The aim of this work is the evaluation of CD44 mRNA and protein expression in canine leukemia. Peripheral blood from 20 acute leukemias (AL) (10 acute lymphoblastic, 6 acute myeloid and 4 acute undifferentiated leukemias), 21 chronic lymphocytic leukemias (CLL) and thirteen healthy dogs were collected. The mRNA expression of all CD44 variants presenting exons 1–5 and/or 16–20 (CD44_{ex1–5} and CD44_{ex16–20}) and CD44 protein were determined by real-time RT-PCR and flow cytometry, using the mean fluorescent index (MFI), respectively. CD44 MFI was significantly higher in leukemic samples compared to controls and a higher expression was found in AL in respect with CLL. No significant differences were found when considering different phenotypic subtypes of AL and CLL. CD44_{ex1–5} mRNA expression was significantly higher in AL compared to controls, whereas there was no difference in CLL compared to controls and AL. CD44_{es16–20} showed the same trend, but without differences among groups. The high CD44 expression found in canine leukemias could be considered a step toward the definition of their molecular features.

Keywords: CD44; Dogs; Acute leukemia; Chronic lymphocytic leukemia

1. Introduction

Cell surface hyaluronan receptor CD44 is a transmembrane glycoprotein encoded by a single gene, involved in leukocyte recirculation and homing, adhesion to extracellular matrix, angiogenesis, cell proliferation and differentiation (DeLisser, 2009). CD44 is encoded by a highly conserved gene of approximately 50–60 kb and composed of a number of constant and variable exons: the former encoding the extracellular globular domain (exons 1–5), the short stem connecting to the cell membrane (exons 16 and 17) and the transmembrane domain (exon 18). The exons 6–15 are variable (v1–10), enlarging the stem on its distal site and forming several distinct CD44 isoforms (Hertweck et al., 2011). The smallest CD44 molecule lacks the entire variable region and it is named CD44 standard (CD44s). CD44s is expressed ubiquitously at high levels in hematopoietic cells. Different combinations of the variable exons create a repertoire of several dozen isoforms that referred to as CD44 variants (CD44v). However CD44v expression is only restricted to a subset of tissues (Liu and Jiang, 2006).

CD44 has been associated to a number of physiological activities although its expression was correlated to tumorigenesis. CD44 was characterized in many tumors mainly stimulating tumor cell proliferation and invasiveness by the recruitment and the activation of surface-associated matrix metalloproteinases (Yu and Stamenkovic, 1999). Moreover, CD44 acts as co-receptors for the activation of growth promoting tumor receptor tyrosine kinases and specific CD44v functions as

tumor cell–surface ligands interacting with endothelial selectins to mediate the initial capture and arrest of circulating tumor cells at a secondary organ (Hertweck et al., 2011).

In hematological neoplasms, CD44 is involved in development and progression by enhancement of apoptotic resistance, invasiveness, as well as regulation of bone marrow homing, and mobilization of leukemia-initiating cells (LIC) into the peripheral blood. In pediatric acute lymphoblastic leukemias (ALL), CD44s and CD44v6 have been identified as prognostic markers: high or low expression patterns have been reported to differentiate prognostic subgroups in ALL. High CD44v6 mRNA levels were correlated with increased risk of relapse (Magyarosy et al., 2001). Also, in acute myeloid leukemia (AML), CD44 signaling plays a pivotal role based on its involvement in differentiation arrest, bone marrow niche dependency of LIC and acquired therapy resistance (Hertweck et al., 2011). Some authors reported a remarkable increase in the complexity of CD44v expression in cells from patients with AML (Bendall et al., 2004). Its activation in acute monoblastic leukemia cells seems to prevent apoptosis (Sansone et al., 2012). In neoplasms of mature cell, as chronic lymphocytic leukemia (CLL), increased serum levels of soluble CD44 and higher expression of CD44 on cell surface are associated with advanced disease, therapy requirement and lower median survival (Eisterer et al., 2004). In particular, CD44 displays an anti-apoptotic effect in CLL cells and a complex of CD44v, CD49d, CD38 and proMMP-9 located on the surface of B-CLL cells play a role in migration and in pro-survival signaling, thus promoting the accumulation of leukemic cells in the tissues in more advanced and progressive diseases (Buggins et al., 2011).

In dogs, acute leukemias (AL) and CLL show different clinical behavior: while CLL generally has an indolent course and a chemotherapeutical approach is required only in cases of progressive disease, ALL and AML show an aggressive behavior and a very poor prognosis. However, both CLL and AL are poorly characterized in dogs and few pathogenic and prognostic studies are available in the literature (Aricò et al., 2013, Comazzi et al., 2011, Giantin et al., 2013 and Usher et al., 2009). To the best of our knowledge, no information is present in the veterinary literature on the role of CD44 in canine hematopoietic tumors.

The aim of this work was the evaluation of CD44 mRNA and protein expression in peripheral blood from dogs with precursor cell (ALL and AML) and mature cell (CLL) leukemia and to investigate possible correlations with different clinical behavior.

2. Materials and methods

Peripheral blood (PB) and bone marrow (BM) samples, collected in EDTA tubes, from dogs suspected of hematopoietic neoplasm were sent to the Department of Veterinary Sciences and Public Health, University of Milan by referring veterinarians for diagnostic purposes.

Part of the sample was used for routine diagnostic procedures and flow cytometric analysis, while the remainder was preserved in polypropylene tubes with 10 parts of RNAlater solution (Life Technologies, Foster City, CA) for total RNA isolation.

PB samples were analyzed with the Sysmex XT-2000iV (Sysmex Corporation, Kobe, Japan). PB and BM smears, when available, were stained with May–Grunwald–Giemsa (Merck KGaA,

Frankfurt, Germany) and used to perform leukocyte differential count and morphological evaluation of blast cells.

Flow cytometric analysis was performed on PB as previously described (Comazzi et al., 2011). The following monoclonal antibodies were used: CD45-PE (clone YKIX716.13, Serotec), CD44-FITC (clone IM7, Pharmingen), CD3-FITC (clone CA17.2A12, Serotec, T cells), CD4-FITC (clone YKIX302.9, Serotec, T-helper and neutrophils), CD8-PE (clone YCATE55.9, Serotec, T-cytotoxic/suppressor cells), CD5-PE (clone YKIX322.3, T-cell), CD21-PE (clone CA21D6 Serotec, mature B cells), CD34-PE (clone 1H6 Pharmingen, precursor cells), CD79a-PE (clone HM57, Serotec, B-cells), CD14-PE (clone TUK 4 Serotec, monocytes), CD11b-PEcy5 (clone M1/70 e-bioscience, myeloid cells), MPO-FITC (clone 2C7, Serotec, myeloid cells). Acquisition was performed with FACSCalibur (Becton Dickinson, San Jose, CA) and analysis was conducted by a commercially available software (Cell Quest, BD). The following clinical, clinical-pathological and immunophenotypical features were used to classify samples:

-AL: moderate to severe anemia and/or thrombocytopenia, leukocyte morphology suggestive of immature or blast cells, more than 20% of blast cells in BM, positivity to CD34, and to CD3, CD5, CD4 or CD8 for T-cell acute lymphoblastic leukemia (T-ALL), to CD21, CD79a for B-cell acute lymphoblastic leukemia (B-ALL), to at least one myeloid marker for acute myeloid leukemia (AML), using the FAB classification (McManus, 2005). Samples were classified as acute undifferentiated leukemia (AUL) when atypical cells expressed only CD34 and CD45.

-CLL: severe lymphocytosis, monomorphic population of mature lymphocytes, positivity to CD3, CD5, CD4 or CD8, for T-cell chronic lymphocytic leukemia (T-CLL) and CD21, CD79a for B-cell chronic lymphocytic leukemia (B-CLL), negativity to serologic titer for Ehrlichia or Leishmania or any other identifiable cause of lymphocytosis.

Thirteen PB samples from healthy dogs were used as controls. All dogs were privately owned and sampled for diagnostic purposes with the informed consent of the owners. A formal approval of the Institutional Committee for Animal Care of the University of Milan was not necessary according to the internal guidelines since samples were sent for diagnostic purposes.

Fifty microliters of cells suspension were used for surface staining for CD44-FITC and CD45-PE. Cells labeled with the appropriate isotype were used as controls. For each PB sample, the mononucleated cells (lymphocytes and neoplastic population) were gated based on morphological properties and CD45 staining, by using either the forward scatter (FSC) versus side scatter (SS) dot plot then the CD45-PE versus SS dot plot, respectively (Fig. 1). For each sample, the CD44 mean fluorescent index (MFI) was calculated as the ratio of MFI of neoplastic cells/MFI of control cells (Advani et al., 2008).

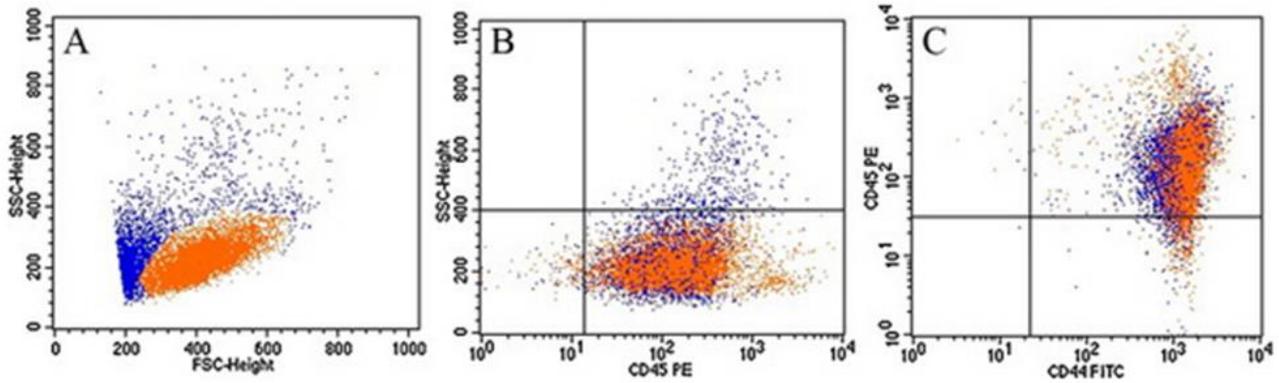


Fig. 1.

Flow cytometric analysis of an acute lymphoblastic leukemia (ALL) sample. Panel A: morphological scatter plot with forward scatter (FSC) versus side scatter (SSC). Panel B: scatter plot with CD45 versus SSC. Neoplastic cells showed a low SSC and a normal to diminished CD45 expression. Panel C: scatter plot with CD44 versus CD45. Neoplastic cells were double positive with high expression of CD44.

Total RNA was isolated from both cell pellet and RNAlater suspension, using the RNeasy Mini Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. To avoid genomic DNA contaminations, on-column DNase digestion with the RNase-free DNase set (Qiagen, Milan, Italy) was performed. Total RNA concentration and quality were measured with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, Germany) and by denaturing gel electrophoresis. First-strand cDNA was synthesized as previously described (Giantin et al., 2013). The cDNA was used as a template for qPCR analysis.

In order to detect all possible CD44 variants, primer design was performed considering exons 1–5 and 16–20, representing CD44 standard exons adjacent to the 5' and 3' site of insertion of variants, respectively. Primer pairs and the appropriate human Universal Probe Library (UPL) probe were selected from canine CD44 mRNA sequence NM_001197022, by using UPL Assay Design Centre web service default parameters. Exons 1–5 region (CD44_ex1–5) was amplified with 5'-AATGCCTTTGATGGACCAAT-3' (forward primer), 5'-TGTCTTCAGGGTTGGTTCTGT-3' (reverse primer), and number #129 human Universal Probe Library (UPL, Roche Applied Science, Indianapolis, IN) probe, while exons 16–20 region (CD44_ex16–20) was amplified with 5'-GGTCCATACCACTCATGGA-3' (forward primer), 5'-GCACCACCTTCTTGACTTCC-3' (reverse primer), and number #130 human UPL probe. Primer specificity was evaluated *in silico* by means of BLAST tool and also experimentally by Power SYBR Green I (Life Technologies, Carlsbad, CA) amplification and melting curve analysis. The amplicon size was 94 and 64 base pairs for CD44_ex1–5 and CD44_ex16–20, respectively. The qPCR (10 μ L final volume) consisted of 1 \times LightCycler 480 Probe Master (Roche Applied Science, Indianapolis, IN), 600 nM forward and reverse primers (Eurofins MWG Operon, Ebersberg, Germany), 100 nM human UPL probe and 2.5 μ L of 40-fold diluted cDNA. qPCR analysis was performed in duplicate in a LightCycler 480 Instrument (Roche Applied Science, Indianapolis, IN) using standard PCR conditions (an activation step at 95 $^{\circ}$ C for 10 min; 45 cycles at 95 $^{\circ}$ C for 10 s and at 60 $^{\circ}$ C for 30 s; a cooling step at 40 $^{\circ}$ C

for 30 s) and LightCycler 480 clear plates (Roche Applied Science, Indianapolis, IN). No template controls and no-reverse transcription controls were included on each plate. Calibration curves, using a 2-fold serial dilution of a cDNA pool, revealed a PCR efficiency of 99.0% and 95.5%, an error value of 0.066 and 0.086, and a dynamic range of about 7 cycles (22.28–29.64 and 23.52–29.77) for CD44_ex1–5 and CD44_ex16–20, respectively. Canine golgin A1 (GOLGA1) was chosen as reference gene (Giantin et al., 2013). Data were analyzed with the LightCycler480 software release 1.5.0 (Roche Applied Science, Indianapolis, IN) by using the fit point method. Messenger RNA relative quantification was performed using the $\Delta\Delta C_t$ method and a cDNA pool including both healthy and pathologic samples as calibrator.

Statistical analysis was performed with SPSS version 19.0. Kruskal–Wallis test was used to compare the gene expression and flow cytometry results for CD44 among healthy dogs and dogs affected by AL and CLL and among different phenotypes of acute leukemia (ALL; AML; AUL). Statistical significance was set at $P < 0.05$ for Kruskal–Wallis test and was set at <0.01 for Mann–Whitney test (post hoc analysis). Grubb's test was used to identify potential outliers.

3. Results and discussion

A total of 41 PB samples were analyzed. Based on clinical, hematological and immunophenotypical data, in 20 dogs the diagnosis was compatible with AL (10 ALL: 7 B-ALL, 3 T-ALL; 6 AML: 5 AML4, 1 AML5; 4 AUL), in 21 dogs with CLL (10 T-CLL, 8 B-CLL, 3 with atypical phenotype: 2 cases of CD3+ CD4– CD8– and 1 case of CD3+ and CD21+).

The MFI of CD44 in control dogs and in leukemia samples is shown in Table 1. In AL, MFI was 400.35 ± 167.39 (median value: 429.03) while in CLL was 240.44 ± 131.44 (median value 235.28). CD44 MFI was significantly higher in both leukemic groups and in all AL and CLL different subtypes when compared to controls ($P < 0.001$). In addition, a higher expression was found in AL compared to CLL ($P = 0.001$). No statistically significant differences were found between different phenotypic subtypes of AL and CLL. However, in CLL a progressive decrease in CD44 expression was found in T-CLL compared to B-CLL and atypical CLL.

Table 1.

CD44 MFI results in peripheral blood lymphocytes and neoplastic cells.

Group	N	CD44 MFI
Control dogs	13	35.07 ± 27.89 (26.69)
Acute leukemia (AL)	20	400.35 ± 167.39*# (429.03)
ALL	10	362.91 ± 178.66* (407.15)
AML	6	450.15 ± 195.52* (459.67)
AUL	4	419.25 ± 88.80* (411.75)
Chronic lymphocytic leukemia (CLL)	21	240.44 ± 131.44* (235.28)
T cell-CLL	10	291.06 ± 157.65* (261.97)
B cell-CLL	8	206.31 ± 75.32* (173.45)
Atypical CLL	3	162.75 ± 120.63* (99.08)

Mean ± standard deviation (median values).

CLL, chronic lymphocytic leukemia; AL, acute leukemia; AML, acute myeloid leukemia; ALL, acute lymphoid leukemia; AUL, acute undifferentiated leukemia.

* $P < 0.001$ vs control group.

$P < 0.003$ vs chronic lymphocytic leukemia.

All acute and chronic leukemia subtype showed an higher CD44 protein expression compare to control dogs and, taken together, AL showed higher expression compare to CLL. No statistically significant differences among different phenotypic subtypes of AL and CLL were found.

Table 1.

CD44 mRNA amount was measured in PB from 6 healthy dogs, 11 AL and 12 CLL. CD44_{ex1-5} mRNA expression was significantly higher in AL compared to controls ($P < 0.01$), whereas there was no difference in CLL compared to controls and AL. CD44_{es16-20} mirrored CD44_{es1-5} behavior, but no differences were found among groups (Fig. 2).

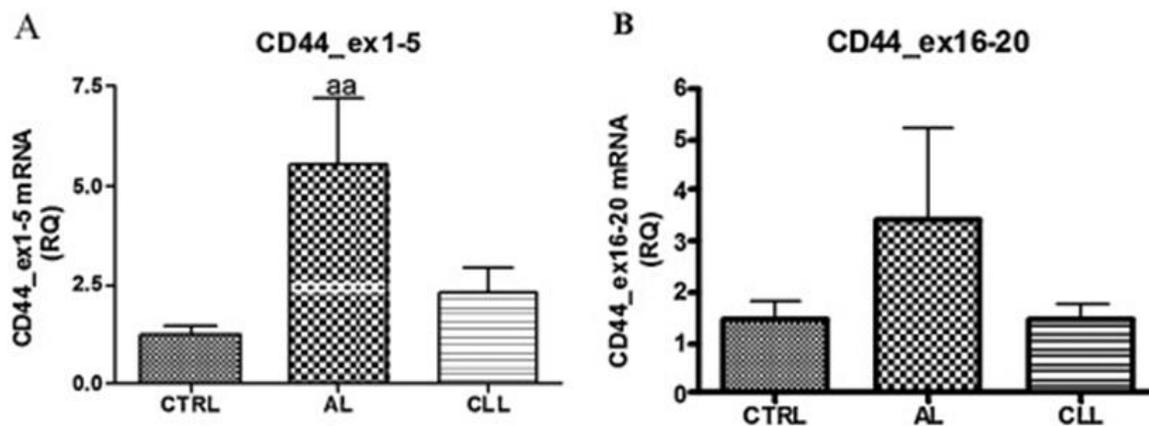


Fig. 2.

CD44 mRNA expression in peripheral blood of control and leukemic samples. aa: $P < 0.001$ vs control group; CTRL: control dogs; AL: acute leukemia; CLL: chronic lymphocytic leukemia.

In this work, we analyzed the mRNA and protein expression of cell surface hyaluronan receptor CD44s in canine leukemias and we found a significantly higher protein expression in AL and CLL compared to control dogs and in precursor cell compared to mature cell neoplasms. These data were confirmed by the qPCR when comparing AL to control dogs. CD44 plays a pivotal role in the development of both myeloid and lymphoid cells mediating the interaction between precursor cells and their niches in the bone marrow, favoring lymphocytes migration, reducing Fas-mediated apoptosis in T helper cells and playing a proliferation-regulation effect in B lymphocytes (Hertweck et al., 2011). As a consequence, altered CD44 expression is crucial in the malignant transformation of different hematopoietic tumors in human medicine. High levels of CD44 were correlated with a poor outcome in childhood ALL and a higher complexity of CD44v was reported in AML cells compared to normal hematopoietic cells (Liu and Jiang, 2006). Interestingly, the block of the interaction between CD44 and its ligand by monoclonal antibodies or vaccination is now considered a possible therapeutical approach in AL (Hertweck et al., 2011). In veterinary medicine, AL is a fatal disease with a very short survival time, due to the massive bone marrow, peripheral blood and other organ involvement. In our study, AL showed the highest expression of CD44, both at mRNA and protein levels and this could partially explain the aggressive behavior of the disease. The presence of an elevated number of CD44 molecules on cell surface could sustain blast cells both in the bone marrow and in the peripheral tissue promoting the interaction with the bone marrow microenvironment, stimulating the homing and the differentiation arrest of LIC in the hematopoietic stem cells niche (Zöller, 2011). Furthermore, CD44 could promote the capture of neoplastic cells on endothelial cells strengthening their binding to hyaluronan and, as a consequence, promoting the tissue invasion. The similar CD44 expression in different AL subtypes suggests that this mechanism is present in blast cells independently of the origin.

In CLL, we also found a significantly higher CD44 MFI compared to control dogs, even if lower than AL. A mechanism of inhibition of apoptosis and increased survival rather than an increased proliferation of neoplastic lymphocytes could explain the more indolent course of canine CLL

compared to AL. A CD44 mediated anti-apoptotic pathway has been demonstrated in human B-CLL (Fedorchenko et al., 2013), but the association between increased expression and prognosis is still undetermined (Fedorchenko et al., 2013 and Herishanu et al., 2011). Our results did not reveal any significant difference in CD44 MFI among different phenotypical subtypes which have been considered of prognostic value in dog (Comazzi et al., 2011), however a definitive conclusion may not be assessed due to the reduced number of cases for each subgroup. mRNA levels of the constant region of CD44, composed by exons 1–5, which encodes for the extracellular globular domain, and exons 16–20, which encodes for transmembrane region and cytoplasmic tail were also analyzed. Both regions showed an over-expression in AL and CLL, but only CD44_ex1–5 were significantly higher in AL compared to control dogs. The different results obtained for mRNA and protein expression could be related to post-transcriptional control, but this mechanism was not investigated in this study. Also, the limited sample number and the high individual variability, particularly in exons 16–20 for both AL and CLL samples may alter this association. In a previous work, we evaluated the MMP-9 mRNA expression in the same samples used in the current study and we found the highest level in CLL (Aricò et al., 2013), but no correlation with CD44 mRNA expression was found (data not shown). These molecules, together with CD38 and CD49d, form a macromolecular complex in neoplastic B lymphocytes in human B-CLL providing a demonstration of the crucial role of the cross-talk between adhesion molecules and pro-angiogenic factors in disease progression (Buggins et al., 2011). The lack of correlation between CD44s and MMP-9 in our previous results could be evidence for a different scenario in canine CLL, however it's important to point out that we analyzed mainly T-CLL and a different interaction between these molecules in neoplastic T lymphocytes cannot be excluded.

Further studies are necessary incorporating a higher number of different AL and CLL subtypes and the evaluation of different CD44 variants, both at mRNA and protein level for a better identification of the role of the hyaluronan receptor in canine leukemias. In conclusion, this is the first work in which CD44 expression was evaluated in canine AL and CLL. In common with the human data, also canine AL and CLL display increased CD44 protein expression compared to control samples. These results, combined with previous works, where c-KIT expression and a role of angiogenic factors, metalloproteinases and their inhibitors were identified in canine leukemia (Aricò et al., 2013 and Giantin et al., 2013) represent a step forward in the definition of the molecular features of these hematopoietic disorders, identify new target molecules and similarities with human leukemias.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Acknowledgement

This work was partially supported by a grant from Ministry of Health (PRIN 2008).

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