Veterinary Immunology and Immunopathology 155 (2013) 117–123

Contents lists available at SciVerse ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

The expression ratio of miR-17-5p and miR-155 correlates with grading in canine splenic lymphoma

Francesca Albonico, Michele Mortarino, Giancarlo Avallone, Gloria Gioia, Stefano Comazzi, Paola Roccabianca *

Dipartimento di Scienze Veterinarie e Sanità Pubblica, Università degli Studi di Milano, Via Celoria 10, 20133 Milano, Italy

ARTICLE INFO

Article history:
Received 10 May 2013
Received in revised form 24 June 2013
Accepted 27 June 2013

Keywords:
MicroRNA
Dog
Splenic lymphoma
FFPE
Grading
RT-qPCR

ABSTRACT

In dogs as in humans, microRNAs (miRNAs) play a key role in normal and neoplastic hematopoiesis regulation. The general miRNA expression framework varies among different stages of development and differentiation of tumors, and miRNAs are widely investigated as new molecular tools for cancer diagnosis and classification. Canine lymphomas are currently classified according with the WHO classification, but a comprehensive grading study of clinical samples is still lacking, and molecular tools for quick grading are not yet available. In the present work, a retrospective study of the expression profile of a panel of miRNAs in canine primary splenic lymphomas was performed. The formalin fixed, paraffin embedded (FFPE) lymphoma samples were accurately classified according with the WHO classification, and were analyzed for miRNA expression using stem-loop TaqMan real time RT-PCR. For each miRNA investigated, relative and absolute quantification were performed after selecting the best housekeeping genes using the NormFinder and geNorm algorithms. The results of this study show a diversity in miRNA expression in low (L) grade lymphomas compared to intermediate-high (I-H) grade lymphomas. The molar ratio between miR-17-5p and miR-155 correlated with WHO grading. These results highlight the potential use of miR-17-5p/miR-155 molar ratio as a new molecular tool for grading of canine splenic lymphomas. The data here reported further support the utility of monitoring miRNA expression in canine hematopoietic malignancies diagnosis and prognosis.

1. Introduction

In the spleen, primary lymphomas occur as nodular or diffuse lesions (Valli et al., 2006; Valli, 2007). It has been estimated that 5.3–29% of all lymphomas in dogs are low-grade and characterized by an indolent clinical course, incomplete response to chemotherapy, but longer survival times compared to aggressive lymphomas (Carter et al., 1986; Fournel-Fleury et al., 1997; Ponce et al., 2010). Primary nodular lymphomas have been described in dogs as a group of mostly low grade tumors deriving from mature B cells (Valli, 2007; Stefanello et al., 2011; Flood-Knapik et al., 2012). Canine lymphomas are currently classified according to the WHO classification (Valli et al., 2011). Histopathological subtypes of canine splenic lymphoma include marginal zone lymphoma (MZL), follicular lymphoma (FL) and mantle cell lymphoma (MCL), which are all of B-cell immunophenotype (Valli et al., 2011). Diffuse primary splenic lymphomas seem less common than nodular lymphomas in dogs while diffuse sinusoidal splenic invasion by leukemias occurs on a regular basis (Workman et al., 2003; Vail and Young, 2007; Valli, 2007). Large B cell lymphoma has been reported in dogs and

* Corresponding author. Tel.: +39 02 50318114; fax: +39 02 50318106.
E-mail addresses: francesca.albonico@unimi.it (F. Albonico), michele.mortarino@unimi.it (M. Mortarino), giancarlo.avallone@unimi.it (G. Avallone), gloria.gioia@unimi.it (G. Gioia), stefano.comazzi@unimi.it (S. Comazzi), paola.roccabianca@unimi.it (P. Roccabianca).

0165-2427/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.vetimm.2013.06.018

seems to develop in the spleen as a multifocal tumor that tends to become diffuse by the time of diagnosis but no data are available on its behaviour. In these dogs primary diffuse involvement of the spleen with severe splenomegaly are characteristic of this rapidly fatal disease (Valli, 2007). Thus, while nodular lymphomas are generally indolent and slow progressive tumors regarded to bear a good prognosis, diffuse lymphomas are mostly associated with a poorer prognosis in dogs as well as in human beings.

MicroRNAs (miRNAs) are a class of small (19–25 nucleotides), non-coding RNAs involved in the negative regulation of gene expression. These molecules bind, through sequence-specific base pairing, complementary regions on the mRNA target, resulting in translational repression or mRNA degradation and gene silencing (Bartel, 2004). MiRNAs are expressed in several organisms and are highly conserved across different species. These molecules are highly specific for tissue and development stage, and play crucial functions in the regulation of important processes, such as development, proliferation, differentiation, apoptosis and stress response (Miska, 2005; Iorio and Croce, 2009). Emerging evidence in humans, suggests that miRNAs play important roles also in the pathogenesis of several types of cancers: some miRNAs may be directly involved in cancer development by controlling cell differentiation and apoptosis, while others may target cancer oncogenes and/or tumor suppressors (Zhang et al., 2007). There are many reports of differential expression of miRNAs between normal and tumoral tissue. Furthermore, miRNA expression profiles vary considerably among different tumor types and are able to reflect the stages of development and differentiation of various tumors (Iorio et al., 2005; Lu et al., 2005; Bloomston et al., 2007; Porkka et al., 2007; Neely et al., 2010). These data suggest a potential use of such molecules as tumoral biomarkers for diagnostic and prognostic purposes in cancer research.

The purpose of the present study was to establish a suitable molecular parameter for quick grading of archival samples of canine primary splenic lymphomas and compare it with the WHO histological classification and grading. This was performed through the expression analysis of a panel of miRNAs in formalin fixed, paraffin embedded (FFPE) samples from indolent nodular and diffuse splenic lymphomas, compared to non-neoplastic splenic samples.

2. Materials and methods

2.1. Lymphoma cases

Canine cases of confirmed primary splenic lymphoma were identified through the medical records and biopsy and necropsy database search of the electronic archives of the Anatomical Pathology service of the School of Veterinary Medicine of the University of Milano, Italy. Twenty-nine cases of primary splenic lymphomas were evaluated and tissues from 7 normal spleens from necropsy investigations were included as controls (Supplementary Table 1). The clinical data of each dog were recorded. Tissue samples were fixed in 10% buffered formalin, routinely processed, and 5 μm sections were stained with haematoxylin and eosin for histopathological evaluation. Lymphomas were classified and graded according to the WHO classification (Valli et al., 2011). As part of the WHO classification, cell size and the number of mitoses were assessed. Lymphomas were classified according with cell size as small (nuclei approximately 1 times the diameter of a red blood cell), intermediate (nuclei approximately 1.5 to < 2 times the diameter of a red blood cell) and large (nuclei approximately ≥2 times the diameter of a red blood cell) (Valli et al., 2011). The number of mitoses was evaluated by counting mitoses per 10 representative, artefact-free, fields at 400× magnification by three pathologists independently. If the mitotic index differed among pathologists a mean of the three counts was calculated.

2.2. Phenotype assessment

For the application of the WHO classification, tumor cell phenotype was assessed. Briefly, 5 μm tissue sections were glued onto polylysine coated glass slides and were dewaxed and rehydrated before immunostaining. Immunohistochemical staining was performed utilizing monoclonal anti-human CD79a-α (Dako, Glostrup, Denmark) at 1:100 and polyclonal anti-human CD20 (Neomarkers, Freemont, CA) at 1:400 for B cells recognition. Polyclonal anti-CD3-ε (Dako, Glostrup, Denmark) was utilized at 1:900 dilution for the identification of the intracellular epsilon chain expressed mostly in T cells. These antibodies recognize epitopes conserved in many species and have been extensively utilized also in dogs (Jubala et al., 2005; Stefanello et al., 2011; Flood-Knapik et al., 2012). Heat induced antigen retrieval was performed by incubation in citrate buffer (pH 6.4) and heated in a microwave oven for 1 min at 900 watt and for 3 min for two times at 750 watt and then cooled at room temperature. Negative controls consisted of substitution of specific antibodies with an isotype-matched, irrelevant monoclonal antibody. Slides with primary antibodies were incubated overnight in a humidified chamber at 4 °C. Secondary detection was performed with the Avidin-Biotin enzyme Complex (ABC kit, Vectastain®, Burlingame, CA, USA) for 30 min. The immunoreaction was visualized with amino-9-ethylcarbazole chromogen (AEC, Kit, Vector, Burlingame, CA, USA). Smears were counterstained with Mayer's haematoxylin for 3 min and cover-slipped with an aqueous mounting media (Glycerine, Sigma–Aldrich®, St. Louis, MO, USA).

2.3. miRNA selection

Ten computationally predicted canine miRNA sequences (*cfa-let-7a*, *cfa-miR-16*, *cfa-miR-17-5p*, *cfa-miR-21*, *cfa-miR-26b*, *cfa-miR-29b*, *cfa-miR-150*, *cfa-miR-155*, *cfa-miR-181a* e *cfa-miR-223*) were obtained from miRBase (http://microrna.sanger.ac.uk). Six of these miRNA genes (*cfa-let-7a*, *cfa-miR-16*, *cfa-miR-21*, *cfa-miR-26b*, *cfa-miR-150*, *and cfa-miR-223*) were investigated as candidate endogenous control (EC) genes based on their previous use as ECs in human and canine hematopoietic cancer studies (Davoren et al., 2008; Merkerova et al., 2008; Mortarino et al., 2010; Gioia et al., 2011). The