Veterinary Immunology and Immunopathology 130 (2009) 59–69

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

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

Research paper

A new subgroup of immunoglobulin heavy chain variable region genes for the assessment of clonality in feline B-cell lymphomas

M. Henrich *, W. Hecht, A.T. Weiss, M. Reinacher

Institut für Veterinär-Pathologie, Justus-Liebig-Universität Giessen, Giessen, Germany, Frankfurter Strasse 96, 35392 Giessen, Germany

ARTICLE INFO

Article history:
Received 28 July 2008
Received in revised form 6 January 2009
Accepted 21 January 2009

Keywords:
Cat
Lymphoma
Clonality
Immunoglobulin
Heavy chain
Complementarity determining region
Framework region

ABSTRACT

In human medicine, PCR-amplification of the complementarity determining region 3 of the immunoglobulin heavy chain genes followed by polyacrylamide gel electrophoresis (PAGE) is an accepted method to assess clonality in B-cell lymphomas and thereby facilitates the differentiation of neoplasias from benign hyperplasias or reactive infiltrates. To generate a basis for the development of a PCR-based assay for the assessment of clonality in feline B-cell lymphomas we analyzed 28 transcripts (cDNA) of the feline immunoglobulin heavy chain variable region genes (*IGHV*). Transcripts were generated using techniques for the amplification of unknown sequences (i.e. the SMART™ RACE and the CapFishing™ technique) as well as primers derived from sequences of the NCBI Trace archive of the cat. Analysis of this archive revealed traces similar to the human *IGHV*-1 and *IGHV*-3 subgroups of genes. By identification of the subgroup-specific leader sequence within the traces, two subgroup-specific primers for this region were designed and used to amplify the heavy chain variable region genes. Using all amplification techniques, transcripts of both subgroups were created and the subgroups were denominated according to their human counterparts as feline *IGHV*-1 and feline *IGHV*-3. By aligning previously described transcripts of the feline *IGHV* genes to our transcripts we were able to assign these to the *IGHV*-3 subgroup; therefore, this study provides the first description of the feline *IGHV*-1 subgroup of genes. On the basis of the *IGHV*-1 and *IGHV*-3 transcripts we developed a PCR-based assay. For each of the two subgroups we used one sense primer derived from the first and one sense primer derived from the third framework region each in combination with a mixture of three antisense primers derived from the fourth framework region. With these four sets of primers, the assay was able to detect monoclonality in 7/10 (70%) cats with histologically and immunohistochemically diagnosed B-cell lymphomas. In two of these cases, monoclonal rearrangement of the *IGHV* genes was only detectable with *IGHV*-1 subgroup-specific primers. Amplification of feline hyperplastic lymphatic tissue only gave results indicative of polyclonal populations. The use of a PCR-based assay in combination with standard techniques for the diagnosis of feline lymphoma is helpful and the characterization of the additional subgroup of feline variable regions genes puts this assay on a broader basis.

1. Introduction

The diagnosis of a lymphoproliferative disease can be challenging. In some cases, the line between reactive and malignant lymphoproliferation cannot be drawn using standard procedures such as histomorphologic or cytomorphologic examination techniques. Discrimination can be achieved by demonstrating characteristics which are shared by all tumor cells but not by morphologically similar non-neoplastic cells. The basis of such features is displayed in the somatic mutation theory of carcinogenesis (Knudson, 1985). One premise of this theory is that cancer

* Corresponding author. Tel.: +49 641 9938201; fax: +49 641 9938209.
E-mail address: Manfred.Henrich@vetmed.uni-giessen.de (M. Henrich).

0165-2427/$ – see front matter. Crown Copyright © 2009 Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.vetimm.2009.01.006

is derived from a single somatic cell that has accumulated multiple DNA mutations. Following this idea, clonality is a basic characteristic of malignancies including lymphomas. Therefore, showing the clonal nature of a lymphoproliferation can help to diagnose a lymphoma.

A feasible method to demonstrate clonality in B-cell lymphomas is the use of the polymerase chain reaction (PCR) to amplify the complementarity determining region 3 (CDR3) of the immunoglobulin heavy chain variable region genes (*IGHV*). This region is formed during recombination of the immunoglobulin genes thereby assembling genes of the variable (VH), diversity (DH) and joining (JH) segment. The newly formed gene segment encodes the variable part of the immunoglobulin heavy chain (Tonegawa, 1983). Initial gene recombination takes place at one chromosome and if no functional gene is generated, rearrangement at the second allele occurs (Waldmann, 1987).

The CDR3 nucleotide sequence is highly variable in both length and composition. The variability in length is achieved by deletion of nucleotides at both ends of the segments (Pascual and Capra, 1991). In addition, insertions of nontemplate encoded nucleotides at the joining sites of the segments are randomly added by the enzyme terminal deoxynucleotide transferase (Waldmann, 1987). Thus, a polyclonal population of lymphocytes is characterized by a high variability in length and composition of the CDR3 whereas a monoclonal lymphocyte population harbors identical CDR3 sequences. Amplification of the CDR3 of a lymphocyte population and subsequent analysis of the PCR product via electrophoresis can demonstrate the presence or absence of CDR3 length variability. Furthermore, this method can be used for the detection of clonality even in formalin-fixed and paraffin-embedded tissue (Wan et al., 1990).

In human medicine, PCR based assessments of clonality in suspected cases of B-cell neoplasia have been frequently described (Aubin et al., 1995; Brisco et al., 1990; Deane and Norton, 1991; Ramasamy et al., 1992; Wan et al., 1992, 1990). To our knowledge, Werner et al. (2005) are the only authors who developed a PCR assay for the diagnosis of feline B-cell lymphomas. The assay is based on the characterization of 24 feline immunoglobulin heavy chain variable region cDNA transcripts and enabled the detection of monoclonal B-cell populations in 15 of 22 (68%) samples from cats initially diagnosed with lymphomas via histology and immunohistology.

In this study, we confirm the results of Werner et al. (2005) and report the discovery of an additional subgroup of feline *IGHV* genes (for definition of subgroup see Lefranc, 2001) suitable for the molecular diagnosis of B-cell neoplasia.

2. Material and methods

2.1. Analysis of the feline IGHV genes from biologic material

For the analysis of the feline *IGHV* genes, snap frozen splenic tissue from routinely necropsied cats was used. To minimize postmortal alterations of mRNA, only animals were included, on which necropsy was performed within 30 min after death. Total RNA was isolated and purified using the Purescript® RNA Purification Kit (Gentra-Systems, Minnesota, USA) according to the manufacturer's instructions. Further isolation and purification of mRNA was performed using the Oligotex® mRNA Kit (Qiagen, Hilden, Germany).

Due to differences in the quality of results, three different methods for the transcription of mRNA into cDNA were used. These were the SMART™ RACE cDNA Amplification Kit (BD Biosciences Clontech, Heidelberg, Germany), the CapFishing™ Full-length cDNA Premix Kit (BioCat, Heidelberg, Germany) and the AMV reverse transcriptase (peQLab, Erlangen, Germany).

The SMART™ RACE (rapid amplification of cDNA ends) cDNA Amplification Kit was used according to the manufacturer's instructions with an add-on of the RNase-Inhibitor RNasin® (Promega, Mannheim, Germany) during cDNA synthesis. Synthesis of cDNA was initiated using an antisense-primer (FeIgMrIV, see Table 1) designed on the basis of the previously published DNA sequence of feline *IGH* μ constant region genes (Cho et al., 1998; GenBank accession No. D89025). The cDNA was amplified according to the 5′-RACE protocol as seminested PCR by use of the Universal Primer A Mix (BD Biosciences Clontech, Heidelberg, Germany) and a gene-specific antisense primer (FeIgMrIII, see Table 1) also derived from the published sequence of feline *IGH* μ constant region genes (Cho et al., 1998). To increase specificity of the products, amplicons with a length above approximately 680 base pairs (bp) were purified from 1% agarose Tris acetate EDTA (TAE) gel using the NucleoSpin® Extract II Kit (Macherey-Nagel, Dueren, Germany) and used as source of a second amplification. This amplification was performed as nested PCR using the Nested Universal Primer A (BD Biosciences Clontech, Heidelberg, Germany) and a gene-specific antisense primer placed upstream of FeIgMrIII (FeIgMrII, see Table 1).

Random hexameres were used to initiate cDNA-synthesis for the 5′-RACE reaction with the CapFishing™ Full-length cDNA Premix Kit. Further amplification was performed using a gene-specific antisense primer (FeIgMrV, see Table 1) combined with the 5′-RACE primer supplied with the kit.

Synthesis of cDNA transcripts using the AMV reverse transcriptase was initiated by oligo-dT-primers and performed following manufacturers recommendations. These cDNA transcripts were further amplified using primers specific for the leader region of the feline *IGHV* genes (see below) combined with the gene-specific antisense primer FeIgMrV (see Table 1).

PCR reactions (for conditions see Table 2) to amplify the cDNA transcripts described above were performed using the Phusion™ High Fidelity DNA-Polymerase (BioCat, Heidelberg, Germany).

Amplicons with a length above approximately 680 base pairs (bp) were purified from 1% agarose TAE gel as described above and ligated with AccepTor™ Vector (Novagen, Merck, Darmstadt, Germany). Due to the proofreading activity of the Phusion™ High Fidelity DNA-Polymerase (BioCat, Heidelberg, Germany) a preceding step of adding 3′A-overhangs using the BioTherm™ DNA-Polymerase (NatuTec, Frankfurt, Germany) was required. After ligation the plasmid DNA was transformed