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Analysis of gene expression in canine idiopathic pulmonary fibrosis ☆

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) in dogs is a rare disease of unknown aetiology, seen in terrier breeds, particularly the West Highland white terrier (WHWT). The aim of this study was to determine pulmonary gene expression in canine IPF in order to gain insights into the pathogenesis of the disease and to identify possible biomarkers. Microarray analyses were conducted to determine gene expression profiles in the lungs of dogs with IPF and control dogs of various breeds. More than 700 genes were identified as having greater than two-fold difference in expression between the two groups. The significant biological functions associated with these genes were related to cellular growth and proliferation, developmental processes, cellular movement, cell to cell signalling and interaction, and antigen presentation. Altered levels of expression were confirmed by quantitative reverse transcriptase PCR for genes encoding chemokine (C–C) ligand (CCL) 2 (+4.9 times), CCL7 (+6.8 times), interleukin 8 (+4.32 times), chemokine (C–X–C) ligand 14 (+3.4 times), fibroblast activation protein (+4.7 times) and the palate, lung and nasal associated protein (PLUNC, −25 times). Serum CCL2 concentrations were significantly higher in WHWTs with IPF (mean 628.1 pg/mL, interquartile range 460.3–652.7 pg/mL) than unaffected WHWTs (mean 344.0 pg/mL, interquartile range 254.5–415.5 pg/mL; $P = 0.001$). The results support CCL2 as a candidate biomarker for IPF in dogs.

Introduction

Canine idiopathic pulmonary fibrosis (IPF) is an interstitial pulmonary disease characterised by progressive fibrosis, leading to end-stage lung disease. The condition is mainly recognised in middle-aged to older terriers, especially the West Highland white terrier (WHWT). Affected dogs generally have a history of progressive dyspnoea and exercise intolerance, with or without coughing (Corcoran et al., 1999; Heikkila et al., 2011). Differentiating IPF from other chronic respiratory diseases is challenging and the definitive diagnosis ultimately relies on histopathology, although thoracic high resolution computed tomography (HRCT) has high specificity (Johnson et al., 2005). Endothelin-1 has good sensitivity and moderate specificity as a serum biomarker (Krafft et al., 2011), but a combination of biomarkers is likely to be more reliable for diagnosing IPF in dogs.

The mechanisms underlying the development of human IPF remain unknown (Coward et al., 2010; Maher, 2012). However, insights into the pathogenesis of IPF have been gained from gene expression analysis of affected pulmonary tissue (Kaminski et al., 2000; Zuo et al., 2002; Kaminski, 2003; Kaminski and Rosas, 2006; Kelly et al., 2006; Studer and Kaminski, 2007). Tissue-wide gene expression analysis using microarrays is a powerful tool to study disease pathways. Bioinformatic tools, such as Ingenuity pathway analysis (IPA), help to analyse these gene expression changes by grouping relevant genes into biological functions or by filtering genes encoding potential biomarkers (Kaminski and Friedman, 2002; Sterling and Danail, 2010).

The aim of the present study was to examine pulmonary gene expression in canine IPF by microarray analysis in an attempt to gain new insights into the pathogenesis of the disease. We hypothesised that there would be differences in gene expression profiles between dogs with IPF and unaffected canine lungs, that genes previously reported as being differentially expressed in the lungs of human beings with IPF would have similar patterns of expression

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in canine IPF and that the gene expression profile would include genes encoding proteins with biomarker potential. To validate this approach for identifying candidate biomarkers, the serum concentration of one protein was measured in dogs with IPF and unaffected dogs.

Materials and methods

Sources of samples

Lung samples from 12 dogs with IPF and 14 control dogs of various breeds were included in this study. Dogs with IPF included WHWTs ($n = 10$; 4 females, 6 males), and two dogs from other terrier breeds (1 male Scottish terrier and 1 male Lhasa Apso), with a median age of 12 years (range 11–15 years). IPF was diagnosed according to Norris et al. (2005), Heikkila et al. (2011) and Syrjä et al. (2013). Control dogs (6 females, 8 males) included Beagles ($n = 3$), Dachshunds ($n = 2$), mixed breed dogs ($n = 3$) and one each of Jack Russell, Yorkshire, Border collie, Belgian shepherd, Newfoundland and Leonberg breeds, with a median age of 6.5 years (range 0.2–14 years). None of these animals had a clinical history or signs of lower respiratory disease and there were no abnormalities of the respiratory system on thoracic radiographs, postmortem examination and histopathology. The reasons for euthanasia in this group of dogs were aggressiveness (3 dogs), nasal tumour (2 dogs), hepatic carcinoma, mammary tumour, intervertebral disc disease, vertebral fracture, degenerative myelopathy, perianal fistula, urinary incontinence and urinary bladder stones (1 dog each); samples were also obtained from one experimental healthy Beagle dog euthanased in a separate study (study protocol approved by the Committee of Experimental Animals of the University of Liège, Belgium, permit number 763, date of approval 13 March 2008).

Serum samples from 10 WHWTs with IPF (3 females, 7 males; median age 11.2 years, range 8–14 years) and 10 healthy age- and breed-matched unaffected (control) dogs (7 females, 3 males; median age 9.6 years, range 3–14 years) were used for chemokine (C–C) ligand 2 (CCL2) serum determination. Diagnosis of IPF was confirmed by HRCT (4 dogs), histopathology (2 dogs) or both (4 dogs). Health status was confirmed on the basis of absence of clinical signs and on the results of clinical examinations, as well as blood gas analysis (10 dogs) and bronchoscopy, bronchoalveolar fluid (BALF) analysis and HRCT (8 dogs). The protocol was approved by the Committee of Experimental Animals of the University of Helsinki, Finland (permit number ESLH-2008-05403, date of approval 27 June 2008).

Collection and processing of lung tissue

Lung samples were obtained within 30 min of euthanasia. In all dogs with IPF, multiple samples were collected from macroscopically abnormal tissue from at least two separate lung lobes. In control dogs, at least two lung samples adjacent to each other were collected. Samples for RNA extraction were either placed in a 1.5 mL cryotube (CM Labs), frozen in liquid nitrogen and stored at −80 °C ($n = 3$) or transferred into in a cryotube containing RNAlater (Ambio), held at 4 °C for up to 24 h and then frozen at −80 °C until further processing ($n = 23$). Samples for histopathological examination were fixed in 10% neutral buffered formalin. Since the lesion distribution is heterogeneous in IPF, samples for RNA extraction and for histopathology were collected from adjacent areas to ensure that microarray analysis would be performed on lesional tissue.

Detection of levels of gene expression by microarray analysis

RNA extraction and quality assessment

Total RNA was isolated from lung samples using the Micro to Mini Total RNA Extraction Kit (Invitrogen). Tissue (~20 mg) was added to a 2 mL Safe-Lock microcentrifuge tube (Eppendorf) containing 350 μL lysis buffer (Invitrogen), 3.5 μL β-mercaptoethanol and a 5 mm stainless steel bead (Qiagen). Samples were disrupted using a Tissue Lyser (Qiagen) by shaking the tube at 30 cycles/s for 1 min. The resulting lysate was centrifuged at 14,000 *g* for 3 min and the supernatant was processed according to the manufacturer's protocol. The RNA was eluted with 40 μL nuclease-free water. DNAse digestion of the RNA solution was carried out using RQ1 RNAse–free DNase (Promega). RNA was stored at −80 °C.

RNA concentration and quality were measured for each sample by spectrophotometry (NanoDrop 1000, Thermo Scientific). RNA samples with a 260 nm:280 nm ratio of 1.8–2.2 and a 260 nm:230 nm ratio of 1.8–2.0 were considered to be suitable for quantitative reverse transcriptase-PCR (qRT-PCR). RNA quality and integrity were further assessed by automated gel electrophoresis (Experion, Bio-Rad). Samples with no or minimal loss of integrity (RNA integrity number $\geqslant 7$) were deemed to be suitable and were used in microarray experiments.

Microarray analysis

Aliquots of RNA (4 ng) from five dogs in each group (5 WHWTs with IPF: 2 females, 3 males, median age 12 years; 5 healthy controls: 2 Beagles, 1 Jack Russell terrier, 1 Yorkshire terrier and 1 Dachshund, all males, median age 4 years) were pooled in equal proportion to form two experimental sets. Gene expression was analysed via hybridisation to the Affymetrix GeneChip Canine Genome 2.0 Array using the one cycle amplification protocol according to the manufacturer's instructions.[1] This canine specific microarray is a second generation oligonucleotide-based single colour array that assesses the expression of 42,869 probe sets, representing >38,000 canine genes (Oyama and Chittur, 2005). The remainder of the RNA from each dog was used for qRT-PCR.

Data analysis

Data were obtained with the high-density Affymetrix GeneChip Scanner 3000. The Affymetrix call detection algorithm was used to assess which probe sets were reliably detected above background on each array. To qualify for further analysis, a transcript had to be evident in IPF or in control tissues, with a probe signal intensity greater than two times the background intensity. Differential expression of transcripts between IPF and control tissues was determined by comparing probe signal intensities using a cut-off of a two-fold change in expression for transcripts that were present in both conditions. Transcripts meeting these criteria were defined as being differentially expressed between the two groups. Transcripts with a negative fold change were down-regulated, whereas transcripts with a positive fold change were up-regulated in IPF tissues compared with control tissues. Transcript identity was achieved with the annotations provided by the chip manufacturer. Unnamed genes and redundant probe sets were excluded from the list.

Ingenuity pathway analysis

Data obtained with the microarray experiment were evaluated by Ingenuity pathway analysis[2] (Kaminski and Friedman, 2002; Sterling and Danail, 2010). Genes from the data set that met the expression value cut-off value of 2 and that were associated with biological functions in Ingenuity's knowledge base were considered for the analysis. A right-tailed Fisher's exact test, was used to calculate a *P* value determining the probability that each biological function assigned to the data set was due to chance alone; significance was designated at $P < 0.05$ with Benjamini–Hochberg multiple testing correction).

IPA Biomarker[3] was used to extract candidate genes on the basis of biological characteristics most relevant to this study. Filters chosen for this study were: (1) there must be at least a two-fold difference in expression of the gene between IPF and control lung tissues; (2) the gene should be known to be expressed in the lungs; and (3) the corresponding protein should be detectable in the blood and/or in BALF.

Detection of levels of gene expression by quantitative reverse transcriptase PCR

Ten genes were selected for qRT-PCR analysis on the basis of their fold changes in gene expression and their potential role in immunological processes and/or fibrosis and/or potential interest as biomarkers: interleukin 8 (IL8), CCL2, CCL7, chemokine (C–X–C) ligand 14 (CXCL14), fibroblast activation protein α (FAP), palate, lung and nasal associated (PLUNC) protein, matrix metalloproteinase (MMP) 1, MMP7, MMP8 and MMP9. mRNA samples from all dogs with IPF ($n = 12$) and all control dogs ($n = 14$) were used for the qRT-PCR experiments. Negative qRT-PCR with the use of RLP13a primers ensured no genomic contamination of the isolated RNA. TBP and RPS18 were used as non-regulated reference genes for normalisation of gene expression (Peters et al., 2007).

The primer and probe sequences were the same as described previously for IL8, CCL2, CCL7, ribosomal protein L13a (RPL13a), ribosomal protein S18 (RPS18) and TATA box binding protein (TBP) (Peeters et al., 2006, 2007) and were designed using GenBank sequences for CXCL14 (XM_847477), FAP (XM_845112.1), PLUNC (XM_847348), MMP1 (XM_546546), MMP7 (NM_001242726), MMP8 (XM_546547) and MMP9 (NM_001003219) (Peeters et al., 2005) (Table 1).

Quantitative RT-PCR was carried out in a two tubes-two enzymes format using a combination of a reverse transcriptase (ImProm-II Reverse Transcription System, Promega) and Hot-Start Taq Master Mix (Qiagen) (Peters et al., 2004). Relative quantification was performed using the ΔC_t method (Livak and Schmittgen, 2001). Mean relative qRT-PCR expression was compared with the Mann–Whitney *U* test using SigmaStat (Systat Software). The level of significance was set at $P < 0.05$.

Measurement of serum CCL2

Blood samples collected in plain tubes were allowed to clot at room for 30 min and then centrifuged at 4 °C for 15 min at 3000 *g* and the serum was stored at −20 °C. CCL2 analysis was performed with a canine sandwich ELISA kit (Canine CCL2/MCP-1 Quantikine, R&D Systems). Data in the two groups were compared

[1] See: http://media.affymetrix.com/support/downloads/manuals/expression_analysis_technical_manual.pdf (accessed 13 August 2013).

[2] See: http://www.ingenuity.com/products/pathways_analysis.html (accessed 13 August 2013).

[3] See: http://www.ingenuity.com/products/ipa/biomarker-filter (accessed 13 August 2013).