Cytokine Genes Single Nucleotide Polymorphism (SNP) Screening Analyses in Canine Malignant Histiocytosis

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Abstract. *In humans, malignant histiocytosis is a tumour-like disease characterised by increasing proliferation of macrophages and reinforced degradation of erythrocytes. High progression of this disease leads to an unfavourable prognosis for the patients, most of them children up to the age of three years. Histological and cytological findings have proposed an important role of aberrant expression of cytokines in histiocytosis. Due to the fact that Bernese Mountain Dogs (BMD) show a predisposition for spontaneously developing malignant histiocytosis, these dogs could possibly be used as a genetic model organism to elucidate the mechanisms of human malignant histiocytosis. Canine cytokine cDNA transcripts of TNFα, Interleukin-1-α (IL-1α) and Interleukin-1-beta (IL-1β) were screened for single nucleotide polymorphisms (SNPs). SNP screening in canine cytokine transcripts for malignant histiocytosis has not been carried out before. Total RNA was isolated from tissue samples from lung, spleen, testis and skin of 17 different dogs (fifteen BMDs, one Collie and one West Highland Terrier). The corresponding cytokine cDNAs were amplified, sequenced and then screened for SNPs. The resulting effects on the protein sequence were analysed. Several BMDs and the West Highland Terrier showed SNPs in the coding sequences which led to missense mutations within the protein sequences of TNFα, IL1α and IL1β.*

Malignant histiocytosis (MH) and Langerhans cell histiocytosis (LCH) are neoplastic diseases which often spontaneously manifest in histiocytes also called Langerhans cells. These histiocytes rapidly change their behaviour to proliferate like metastases with undetermined pathogenesis and heterogeneous diagnostic findings (1).

Due to aberrant immune response, these histiocytes, normally present in lung, liver, spleen, bones, brain, lymph nodes, skin and other epithelia, transform into highly mobile and infiltrating macrophages, which affect many different organs forming neoplasms with an enhancement of phagocytosis of erythrocytes. The clinical spectrum of this disease is wide with variable degrees of malignancy. However, histopathological findings of granulomas organised in clusters are uniform within the involved tissues (1, 2). The progressive course of histiocytosis is characterised by fast spreading symptoms and unfavourable prognosis, and is particularly often lethal in infants and children. Histiocytosis is not referred to as cancer by definition, although manifestations of this disease behave like tumour diseases, *e.g.,* metastasising and an aggressive invasiveness of growth. Histiocytosis is defined as a disorder of the immune system with vague understanding of aetiology and pathogenesis (1).

As abundantly described in the literature, in many cases dogs and humans share the genetic pathways for the development of neoplastic diseases (3-5). Dogs can be used as an animal model to unravel the mechanisms of malignant histiocytosis as well as LCH. Most clinical and pathological features of canine histiocytosis resemble those of Langerhans cell histiocytosis in humans. In particular, there is a breeding predisposition for this disease observed in Bernesese Mountain Dogs (BMD) (6-8). During the malignant histiocytosis disease, LCH cells and T cells produce large quantities of cytokines, raising a cytokine "storm" (9). Screening for single nucleotide polymorphism (SNP) within the nucleotide sequence of the responsible genes could be an instrument for revealing the cause of the disease. Cytokine genes are abundantly expressed in histiocytes and have been proposed to play a major role in pathogenesis. Due to the fact that the pro-inflammatory cytokines IL-1 (IL-1α and IL-1β) and the tumour necrosis factor-α (TNFα) are described to play a major role as autocrine growth

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Table I. *Detected SNPs and insertion in screened cytokine coding sequences.*

Transcript	Exon	Sample (tissue)	Codon	Substitution	Amino acid exchange
TNFα	4	2 (lung)	206	GAT → GGT	Asp → Gly
IL-1α	3	2 (lung)	23	TTC → TCC	Ser → Pro
	4	2 (lung)	47	TGC → CGC	Cys → Arg
IL-1α Exon 5 del	4	9 (skin)	43	CTT → CCT	Leu → Pro
IL-1β	5	5 (liver)	113	GAT → GGT	Asp → Gly
	5	1 (liver)	124	CAG → AAG	Gln → Lys
	7	4 (liver)	228	TCT → CCT	Ser → Pro
	7	4 (liver)	234	TAC → CAC	Tyr → His

factors in many oncogenic malignancies (2, 10-12), it is interesting to focus on gene expression and SNP screening. Additionally, IL-1α/β were also described to be involved in the regulation of the immune system, fever proteins and inflammationary processes (9, 13).

In order to clarify whether SNPs in canine cytokine transcripts exist, *TNFα* and *IL-1α* and *IL-1β* cDNAs derived from various tissues of dog breeds both with and without a known predisposition for the development of MH were screened. Cytokine cDNAs were derived from healthy lung, skin, liver, spleen and testis tissues from different breeds including BMDs, West Highland Terriers and Collies.

Materials and Methods

Spleen, lung, liver, skin and testis tissue from BMDs, Collies and West Highland Terriers were provided by the Small Animal Clinic, University of Veterinary Medicine, Hanover, Germany. Canine cytokine cDNA transcripts were screened and synthesised by total RNA from 17 different individuals (15 BMDs, one Collie and one West Highland Terrier).

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) protocol for animal tissues and proteinase K digestion. *RNase*-Free *DNase* Set was used for preventing genomic DNA contamination by *DNase* I digestion of the total RNA. For cDNA 3'-RACE synthesis up to 5 μg of total RNA was set as template for reverse transcription (SuperScript II RT, Invitrogen) using AP2 poly-T adaptor primer ($AAGGATCCGTCGACATCT_{17}$). The adjacent RT-PCRs for cytokine cDNA screening were carried out with the following primer pairs. *TNFα*: primer pair TNFaUP / TNFaLO (5'AGCCCCTCTCAGAAGCAGAC'3 / 5'GTCATCGGG GTCTCACATCC'3). *IL-1B*: primer pair ILbUP / ILbLO (5'TTCAGGTTTCTAAAGCAGCCAT'3 / 5'TTAGCAGTGAT TTAGGGAAGGC'3). *IL-1A*: primer pair ILaUP / ILaLO (5'ACAAAAGGCGAAGTAGTCTG'3 / 5'TGTTAGTGTCGGT TCCATTAG'3). An 1.5% agarose gel electrophoresis was performed to separate the PCR products, the fragments were recovered with the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). The PCR products were cloned in the pGEM-T Easy Vector System (Promega, Madison, WI, USA) and sequenced in forward and reverse direction for verification (MWG-Biotech AG, Ebersberg, Germany). The protein sequences were deduced by in silico analyses. Identity comparison of the proteins and respective cDNA contigs were done by Lasergene software (DNAStar, Madison, USA) and miscellaneous canine and human cytokine sequences from the NCBI database (accession numbers AY423389, AF047011, AF322077, AF322078, NM_001037971).

Results

Of the 17 individuals screened, eight cytokine transcripts showed nucleotide substitutions leading to amino acid exchanges within the deduced proteins of TNFα, IL-1α and IL-1β (Table I). SNPs causing no amino acid exchange were not taken into account for further analysis.

A BMD lung sample showed one nucleotide substitution affecting *TNFα* exon 4 codon 113 (GAT → GGT, Asp → Gly).

For *IL-1α*, the complete canine IL-1α coding sequence and the sequence for the alternative *IL-1α Exon 5 del splice* variant were amplified (14). The complete *IL-1α* coding sequence of one lung sample (BMD) showed substitutions in exon 2 codon 23 (TTC → TCC, Ser → Pro) and exon 4 codon 47 (TGC → CGC, Cys → Arg). One substitution was found in the *IL-1α Exon 5 del splice* variant transcript in a skin sample (West Highland Terrier). The transcript showed one nucleotide substitution in exon 4 codon 43 (CTT → CCT, Leu → Pro).

Three *IL-1β* transcripts derived from liver samples showed nucleotide substitution in the canine exons. Two samples (BMD) each showed one nucleotide substitution in the coding sequence of exon 5, affecting codon 113 (GAT → GGT, Asp → Gly) and codon 124 (CAG → AAG, Gln → Lys), respectively. One sample (BMD) showed two substitutions each in exon 7 codon 228 (TCT → CCT, Ser → Pro) and codon 234 (TAC → CAC, Tyr → His).

Discussion

Cytokines are considered to play a major role in the pathogenesis of malignant histiocytosis. In the present study, we focused on SNPs in the canine coding sequences of the cytokines *TNFα*, *IL-1α* and *IL-1β*, causing amino acid exchanges in the deduced protein sequences. With the

exception of two samples (West Highland Terrier and Collie), all samples were taken from BMDs, known to be frequently affected by malignant histiocytosis.

IL-1α and *IL-1β* both belong to the same gene family of *Interleukin-1* and are translated as precursor proteins with a molecular weight of 31 kDa. They are expressed in various cell types, *e.g.,* activated monocytes, macrophages and lymphocytes. The processing of proIL-1α and proIL-1β by cellular proteases results in a mature form of the protein of approximately 17 kDa. Both proteins induce cell signalling pathways upon binding with a membrane-bound receptor IL-1R1, including the NFκB inducing kinase (NIK) and three distinct MAP kinase cascades (13, 15). These switched pathways activate different transcription factors, *e.g.,* NFκB, AP1 and CREB for regulation of immediate early genes vital to inflammatory and immune responses (13). Intracellular proIL-α is fully active and is cleaved by Ca^{2+}-dependent membrane associated cysteine proteases called calpains. ProIL-α is cleaved to IL-1α propiece and mature IL-1α, which is released to the extra cellular compartment (15, 16).

The *IL-1α* transcript (BMD sample 2 / lung tissue) in the present study revealed two amino acid exchanges at position 23 and 47 within the deduced protein sequence of the IL1-1α propiece. At position 23, the polar amino acid serine was substituted by unpolar proline. At position 47, the polar amino acid cysteine was exchanged with positively-charged arginine. Cysteines are able to form disulfide bridges, which are often modified by methylation. Both 32 kDa proIL-1α and the 16 kDa IL-1α propiece are able to bind to nuclear DNA. ProIL-1α is commonly found in the cytoplasm and after myristoylation it binds to the membrane (17).

A SNP in the *IL-1α Exon 5 del* splice variant from skin (Western Highland Terrier sample 9) showed one amino acid exchange at position 43 within the deduced protein isoform IL-1α. Unpolar leucine is substituted to unpolar proline. Due to deletion of exon 5, the calpain cleavage site is lacking and calpain is unable to cleave the mature protein. These splice variant transcripts were mainly detected in macrophages and synovial membrane tissue of dogs, cats and pigs at various expression levels (14).

Pro IL-β remains in the cytoplasm until it is cleaved by the cysteine proteinase interleukin 1β converting enzyme (ICE) to the 16 kDa IL-1β propiece and the biologically active 17 kDa mature IL-1β protein. Either proteins can be myristoylated and are able to be bound to the cell membrane or to be transported out of the cell (14, 15). In the *IL-1β* transcript (BSH sample 1), one SNP showed an amino acid substitution in the deduced canine mature IL-1β sequence. At position, 124 glutamine was substituted by lysine within a β-strand motif of the protein (18-20). At position 228 and 234, two SNPs (BSH sample 4) caused two amino acid exchanges in the deduced canine mature IL-1β also within a β strand motif. At position 228, serine was exchanged with proline and at position 234 tyrosine was exchanged with histidine. Regarding the tyrosine exchange at position 234 the motif asparagine-tryptophan-tyrosine is highly conserved in different species, *e.g.,* human, mouse, rat, cattle and rabbit. However side-directed mutagenesis in this peptide motif of human IL-1β changing the tyrosine residue (Tyr 237) to phenylalanine did not affect the protein binding affinity to its receptor (19).

At position 113, one SNP (BSH sample 5) led to an amino acid exchange in the deduced propiece IL-1β protein. Position 113 covers the last amino acid in the sequence. Hydrophilic aspartic acid is exchanged with unpolar glycine. The aspartic acid – alanine side (canine amino acid position 113 - 114) was determined to be cleaved by cysteine proteinase interleukin β converting enzyme (ICE). Interestingly, all SNPs in the IL-1β transcripts were exclusively detected in liver samples.

The pleiotropic cytokine TNFα is primarily secreted by stimulated macrophages. It additionally acts as a potent pyrogen when stimulated by IL-1. Further on, it plays an important role in abundant cellular signal transduction processes during immune response and inflammation. It can induce cell death in certain tumour cells. TNFα exists in two forms: a soluble form of 157 amino acids (17 kDa), cleaved at amino acid position 76 and 77 by ADAM17 and as a type II membrane protein of 233 amino acids (26 kDa) (21). One SNP in the canine TNFα transcript (BSH sample 2 / lung tissue) led to an amino acid exchange in the deduced membrane binding region of TNFα. At position 206, the amino acid aspartic acid was exchanged with glycine.

SNPs within the promoter and or enhancer region of the cytokine genes IL-1 α/β and TNFα, which play an important role in the development of diseases, *e.g.,* cancer, Alzheimer disease, sepsis and rheumatoid arthritis, were described (22-24). For malignant histiocytosis, the data regarding SNP screening in canine cytokine transcripts is currently scarce. Our results showed several SNPs within the cytokine coding sequence of mRNA isolated from BMD tissue samples. These SNPs led to missense mutations causing changes of amino acid sequences within the deduced protein. Due to the fact that the BMDs show a genetic predisposition for malignant histiocytosis, these dogs could be used to elucidate the genetic mechanisms of malignant histiocytosis and to elucidate in further studies if the found SNPs play a role in the pathogenesis of this disease.

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References

1 Tazi A, Soler P and Hance AJ: Adult pulmonary Langerhans' cell histiocytosis. Thorax *55(5)*: 405-416, 2000.

2 Kannourakis G and Abbas A: The role of cytokines in the pathogenesis of Langerhans cell histiocytosis. Br J Cancer Suppl *23*: S37-40, 1994.

3 Ostrander EA, Galibert F and Patterson DF: Canine genetics comes of age. Trends Genet *16(3)*: 117-124, 2000.

4 Ostrander EA and Kruglyak L: Unleashing the canine genome. Genome Res *10(9)*: 1271-1274, 2000.

5 Patterson DF: Companion animal medicine in the age of medical genetics. J Vet Intern Med *14(1)*: 1-9, 2000.

6 Ramsey IK, McKay JS, Rudorf H and Dobson JM: Malignant histiocytosis in three Bernese mountain dogs. Vet Rec *138(18)*: 440-444, 1996.

7 Nolte I and Nolte M: Praxis der Onkologie bei Hund und Katze. Stuttgard, Enke Verlag, pp. 143-144, 2000.

8 Affolter VK and Moore PF: Localized and disseminated histiocytic sarcoma of dendritic cell origin in dogs. Vet Pathol *39(1)*: 74-83, 2002.

9 Egeler RM, Favara BE, van Meurs M, Laman JD and Claassen E: Differential *in situ* cytokine profiles of Langerhans-like cells and T cells in Langerhans cell histiocytosis: abundant expression of cytokines relevant to disease and treatment. Blood *94(12)*: 4195-4201, 1999.

10 Tazi A, Moreau J, Bergeron A, Dominique S, Hance AJ and Soler P: Evidence that Langerhans cells in adult pulmonary Langerhans cell histiocytosis are mature dendritic cells: importance of the cytokine microenvironment. J Immunol *163(6)*: 3511-3515, 1999.

11 Arico M and Danesino C: Langerhans' cell histiocytosis: is there a role for genetics? Haematologica *86(10)*: 1009-1014, 2001.

12 Arico M: Langerhans cell histiocytosis: Too many cytokines, not enough gene regulation? Pediatr Blood Cancer 2006.

13 Stylianou E and Saklatvala J: Interleukin-1. Int J Biochem Cell Biol *30(10)*: 1075-1079, 1998.

14 Straubinger AF, Viveiros MM and Straubinger RK: Identification of two transcripts of canine, feline, and porcine interleukin-1 alpha. Gene *236(2)*: 273-280, 1999.

15 Dinarello CA: Biologic basis for interleukin-1 in disease. Blood *87(6)*: 2095-2147, 1996.

16 Kobayashi Y, Yamamoto K, Saido T, Kawasaki H, Oppenheim JJ and Matsushima K: Identification of calcium-activated neutral protease as a processing enzyme of human interleukin 1 alpha. Proc Natl Acad Sci USA *87(14)*: 5548-5552, 1990.

17 Stevenson FT, Bursten SL, Fanton C, Locksley RM and Lovett DH: The 31-kDa precursor of interleukin 1 alpha is myristoylated on specific lysines within the 16-kDa N-terminal propiece. Proc Natl Acad Sci USA *90(15)*: 7245-7249, 1993.

18 Priestle JP, Schar HP and Grutter MG: Crystal structure of the cytokine interleukin-1 beta. Embo J *7(2)*: 339-343, 1988.

19 Priestle JP, Schar HP and Grutter MG: Crystallographic refinement of interleukin 1 beta at 2.0 A resolution. Proc Natl Acad Sci USA *86(24)*: 9667-9671, 1989.

20 Eisenberg SP, Brewer MT, Verderber E, Heimdal P, Brandhuber BJ and Thompson RC: Interleukin 1 receptor antagonist is a member of the interleukin 1 gene family: evolution of a cytokine control mechanism. Proc Natl Acad Sci USA *88(12)*: 5232-5236, 1991.

21 Beutler B and Cerami A: The biology of cachectin/TNF – a primary mediator of the host response. Annu Rev Immunol *7*: 625-655, 1989.

22 Dennis RA, Trappe TA, Simpson P, Carroll C, Huang BE, Nagarajan R, Bearden E, Gurley C, Duff GW, Evans WJ, Kornman K and Peterson CA: Interleukin-1 polymorphisms are associated with the inflammatory response in human muscle to acute resistance exercise. J Physiol *560(Pt 3)*: 617-626, 2004.

23 Zienolddiny S, Ryberg D, Maggini V, Skaug V, Canzian F and Haugen A: Polymorphisms of the interleukin-1 beta gene are associated with increased risk of non-small cell lung cancer. Int J Cancer *109(3)*: 353-356, 2004.

24 Correa PA, Gomez LM, Cadena J and Anaya JM: Autoimmunity and tuberculosis. Opposite association with TNF polymorphism. J Rheumatol *32(2)*: 219-224, 2005.

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