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SUSCEPTIBILITY AND RESISTANCE TO CANINE LEISHMANIOSIS IS ASSOCIATED TO POLYMORPHISMS OF THE CANINE TNF-α GENE

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The prevalence of canine leishmaniosis (CanL) infection in an enzootic area is considerably higher than the overall prevalence of the disease, suggesting a role of host genetics related to the outcome of the disease. It is accepted that one determining factor for the outcome of CanL is the type of the triggered immune response, which seems to be genetically determined. TNF-α is a cytokine which plays a crucial role during the immune response against *Leishmania* parasites. In the present study a case-control study with 20 resistant and 20 susceptible dogs was performed. The distribution of breeds was equal in both groups. By Sanger method the nucleotide sequence upstream the Open Reading Frame of the canine TNF-α gene was determined and four polymorphisms were identified (-40 C/A, -1134 T/G, -1150 T/C και -1243 C/G). Statistical analysis showed that the polymorphism TNF-α -40 C/A is correlated with susceptibility to CanL, while the polymorphism TNF-α -1243 C/G is correlated with resistance to CanL. Further statistical analysis, regarding the possible correlation of gender as well as clinical manifestations of the disease with the above-mentioned polymorphisms of the TNF-α gene, showed no significant findings. Further analysis of the above polymorphisms, as well as identification of more polymorphisms in candidate genes, is required to provide a better understanding of the complex underlying immune response in CanL.

Canine leishmaniosis (CanL) is one of the major zoonoses affecting millions of dogs globally and causing life threatening disease (1-2).

CanL is a multisystemic disease with a variety of clinical signs/manifestations, the commonest being skin disease, local or generalized lymphoadenomegaly, progressive loss of body weight, ocular lesions, liver and spleen enlargement, renal disease, epistaxis, onycogryphosis and lameness (1, 3-4). Atypical forms include monoclonal gammopathy, chronic colitis, haemostatic alterations and disorders of the cardiovascular, respiratory and

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musculo-skeletal systems (5). Recent epizootical studies in such areas have confirmed that CanL spreads quickly and extensively among the dog population with a prevalence involving as much as 67%–80%. But high prevalence of infection is accompanied by a considerably lower rate of apparent clinical disease, suggesting a role of host genetics related to the outcome of the disease (1-2).

To date, it has not been clarified which mechanisms in dogs are responsible for resistance or susceptibility (2). One determining factor for the outcome of CanL is the type of the triggered humoral and cellular immune response, which seems to be genetically determined (2-3).

The cytokines IFN-γ, TNF-alpha and IL-12, released in the Th1 immune response induce macrophage activation and NO killing of parasites. In contrast, disease susceptibility which is associated with the appearance of clinical disease is related to the Th2 immune response, which is associated with the production of IL-4, IL-5, IL-6, IL-10 and IL-13 (1, 6-9).

TNF-α is a pleiotropic cytokine secreted by a variety of cells, which exhibits a major role in the initiation and regulation of inflammation, metabolism and immunity responses (10-11). Studies on mice have confirmed that TNF-α is critical for the early control of leishmaniosis and survival of the host (12-14).

To date, there has been increasing evidence that genetic variation within the TNF locus is important in determining susceptibility or severity to certain diseases, and several single nucleotide polymorphisms (SNPs) within the human TNF-a gene sequence, especially those within the promoter region, have been identified and associated with the pathogenesis of a wide range of autoimmune and infectious diseases.

The aim of this study is to identify polymorphisms within the canine TNF-α gene and to correlate them with susceptibility or resistance to CanL.

MATERIALS AND METHODS

Animal cohort and DNA extraction

The case-control study included 40 dogs from 15 different breeds. In order to avoid breed effect, the distribution of breeds to each group of animals was the same (Table I). All dogs were living in Greece.

Table I. *Breed distribution among the two animal groups.*

Breed	Group A	Group B
Alaskan malamute	1	1
Belgian shepherd (Groenendal)	2	2
English setter	2	2
Boxer	1	1
English pointer	1	1
Labrador retriever	1	1
Cocker spaniel	1	1
Doberman pinscher	2	2
German shepherd	2	2
Siberian husky	1	1
Rottweiler	2	2
Collie	1	1
Ghekas (Greek pointer)	2	2
Kurtzhaar	1	1

Group A consisted of 20 healthy dogs (control group - resistant dogs). They were selected among animals over 6 years of age, which were taken to veterinarian hospitals for regular health checks and had never developed any clinical signs of CanL. The *Leishmania* infection was verified by positive parasitological and/or positive anti-*Leishmania* antibody titre. Moreover, the dogs did not exhibit clinical signs of any other concurrent diseases with potent immunosuppressive effect. Finally, the dogs were seronegative (spot ELISA) for *Ehrlichia canis* and *Dirofilaria immitis*.

Group B (susceptible dogs) consisted of 20 dogs with CanL, which had developed clinically severe disease and/or were experiencing continuous relapses. These dogs were from the same breeds and geographical region to those of group A. The diagnosis of CanL was identified by positive parasitological and/or by positive anti-*Leishmania* antibody titre. Moreover, the dogs did not exhibit clinical signs of any other concurrent diseases with potent immunosuppressive effect. Finally, the dogs were seronegative (spot ELISA) for *Ehrlichia canis* and *Dirofilaria immitis*.

Genomic DNA was extracted from 0.3 mL of peripheral whole blood of the examined animals according to the manufacturer's manual ("PUREGENE™ DNA PURIFICATION SYSTEM", Gentra Systems, Minneapolis, MN, USA).

Animal genotyping

An intergenic region of approximately 2150 bp, located between the TNF-β and TNF-α genes (positions

144214 – 146363) was derived from a *Canis familiaris* BAC clone (GenBank accession number AY423389). Three primers were designed using the IDT OligoAnalyzer 3.1 software (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/), targeting the proximal region (promoter) upstream of the translation initiation site of the canine TNF-α gene. More specifically, a semi-nested PCR was applied for amplification of a 1329 bp upstream the ORF (Open Reading Frame) of the canine TNF-α.

All amplifications were performed in a TAKARA Thermal Cycler. The primers used in the first PCR assay were TNF-α-F1 (sense), 5′-CAGGGGTGAGGCAGCCAGACT-3' and TNF-α-R (antisense), 5′- TCCTCGGCCAGCTCCACGT -3′. The 25 μl final volume reactions consisted of 1 μl genomic DNA extract, 0.2 μM of each primer, 0.2 μM dNTPs, 0.6 U High fidelity PCR enzyme mix (K0191, Fermentas, Life Sciences) and 1x High Fidelity PCR Buffer with MgCl2 (10x). Cycling conditions consisted of a pre-heat step of 94°C for 7 minutes, followed by 43 cycles of 95°C for 30 sec, 65°C for 30 sec and 72°C for 75 sec. A final step of 72°C for 3 min allowed for complete extension of products. The semi-nested PCR was performed using the internal primer TNF-α–F2 (sense), 5′-AGAGCCCAAGGTCTGCAGGAGTG-3′ and the TNF-α-R. The 25 μl final volume reactions consisted of 1 μl of the product from the first PCR, 0.2 μM of each primer, 0.2 μM deoxynucleoside triphosphates (dNTPs), 0.6 U High fidelity PCR enzyme mix (K0191, Fermentas, Life Sciences) and 1x High Fidelity PCR Buffer with MgCl2 (10x). Cycling conditions consisted of a pre-heat step of 94°C for 7 min, followed by 32 cycles of 95°C for 30 sec, 58°C for 30 sec and 72°C for 20 sec. A final step of 72°C for 3 min allowed for complete extension of products.

All PCR products were analysed by electrophoresis on 1.2% agarose gels, stained with ethidium bromide and visualised on a UV transilluminator (Fig. 1). Amplicons of the desired size were excised with a clean blade and gel extracted using the Nucleospin Gel extraction kit II (Macherey-Nagel, Germany) according to the manufacturer's manual.

Double-strand sequencing of purified PCR products was performed on an ABI 3100 apparatus (Applied Biosystems). Genotyping analysis in regard to the promoter region polymorphisms was accomplished by direct sequencing of the 1329 bp PCR product and multiple alignment of the derived sequences to the reference sequence, using Clustal. For all the examined samples, a final 1300 bp region upstream the ORF was determined.

TFSearch web server analysis (http://www.cbrc.jp/research/db/*TFSEARCH*.html) was used for the prediction of potential transcription factor binding sites within the vicinity of the identified SNPs.

Statistical analysis

Computation was carried out with Statistical Package for the Social Sciences (SPSS, version 15.0, SPSS Inc., Illinois, USA) and GenePop version 4.0.10 (http://genepop.curtin.edu.au/). POPGENE version 4.0.10 was used for calculating genotypic as well as allelic frequency distributions and the deviations from Hardy-Weinberg equilibrium. Hardy Weinberg equilibrium of alleles at individual loci and comparisons of genotype frequencies between group A and group B were assessed by *chi*-square statistics. Linkage disequilibrium (LD) was determined for the pair of markers using LinkDos program (15). LD was calculated to investigate the strength of allelic association between pairs of markers in group A as well as in group B. Probability (p) values of <0.05 were considered statistically significant.

RESULTS

A region of 1329 bp located upstream the ORF of the canine TNF-a gene was amplified (Fig. 1).

The analysis of a 1300 bp region located upstream the ORF of the canine TNF-a gene, encompassing the TNF-a promoter, in 40 dogs revealed a total of 4 SNPs which are shown in Table II.

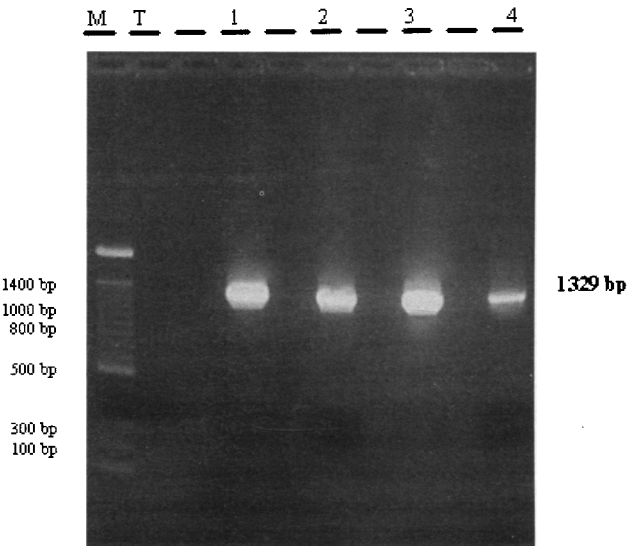

Fig. 1. *PCR products analysed by electrophoresis on 1.2% agarose gels, stained with ethidium bromide and visualised on a UV transilluminator. The band of 1329 bp represents the amplified PCR product. M: DNA ladder, T: negative control and 1 - 4: PCR products of four dogs.*

Table II. *Polymorphic sites within the canine TNF-a promoter region. Positions are numbered relative to the transcription start of the canine TNF-a gene.*

Position	-40	-1134	-1150	-1243
SNP	C/A	T/G	T/C	C/G

These four SNPs were analysed in order to identify genetic factors associated with resistance or susceptibility to CanL. Statistical analysis showed that there is a statistically important difference in the presence of SNP -40 C/A between group A (resistant dogs) and group B (susceptible dogs) ($p<0.000$ by Pearson), since the percentage of presence for this SNP is statistically higher in group B. Consequently, SNP -40 C/A may be a possible infection susceptibility factor (Table III).

Regarding the SNP TNF-α -1134 T/G and the SNP TNF-α -1150 T/C, no statistically significant association was found between the two animal groups ($p = 0.182$ by Fisher and $p = 0.487$ by Fisher, respectively).

Statistical analysis showed that there is a statistically important difference in the presence of SNP -1243 C/G between group A (resistant dogs) and group B (susceptible dogs) ($p<0.006$ by Pearson), since the percentage of presence for this SNP is statistically higher in group A. Consequently, SNP -1243 C/G may be a possible infection-resistant factor (Table III).

There were no significant interactions of TNF-α polymorphisms and gender of the dogs (Table IV).

None of the four TNF-α polymorphisms was significantly associated with the appearance of skin lesions, enlargement of lymph nodes, ocular manifestations or cachexia (Table V).

According to statistical analysis, all loci were in Hardy–Weinberg disequilibrium for both populations ($p>0.05$). Weak Linkage Disequilibrium ($p<0.05$) was observed between all pairs of markers

Table III. *Genotype distribution and allele frequencies of TNF-α gene SNPs in Groups A and B.*

	Genotypes		**Alleles**		
	Group A	**Group B**		**Group A**	**Group B**
SNPs	**n (%)**	**n (%)**		**n (%)**	**n (%)**
TNF-α -40 C/A					
C/C (%)	16 (80)	1 (5)	All 1 C	36 (0.90)	12 (0.30)
C/A (%)	4 (20)	10 (50)	All 2 A	4 (0.10)	28 (0.70)
A/A (%)	0 (0)	9 (45)		p=0.0000	
p=0.000					
TNF-α -1134 T/G					
T/T (%)	19 (95)	15 (75)	All 1 T	39 (0.975)	35 (0.875)
T/G (%)	1 (5)	5 (25)	All 2 G	1 (0.025)	5 (0.125)
G/G (%)	0 (0)	0 (0)		p=0.2021	
P=0.182					
TNF-α -1150 T/C					
T/T (%)	18 (90)	20 (100)	All 1 T	38 (0.95)	40 (1)
T/C (%)	2 (10)	0 (0)	All 2 C	2 (0.05)	0 (0)
C/C (%)	0 (0)	0 (0)		p=0.4953	
p=0.487					
TNF-α -1243 C/G					
C/C (%)	10 (50)	18 (90)	All 1 C	29 (0.725)	38 (0.95)
C/G (%)	10 (50)	2 (10)	All 2 G	11 (0.275)	2 (0.05)
G/G (%)	0 (0)	0 (0)		p=0.0125	
p=0.006					

Table IV. *Correlation of the TNF-a SNPs with gender.*

SNPs	Genotypes	Group A (resistant dogs)		Group B (susceptible dogs)	
		♂	♀	♂	♀
TNF-α -40 C/A	CC AC+AA	6 (37.5%) 3 (75%)	10 (62.5%) 1 (25%)	1 (100%) 8 (42.1%)	0 (0%) 11 (57.9%)
		p = 0.285 by Fisher		p = 0.450 by Fisher	
TNF-α -1134 T/G	TT TG+GG	9 (47.4%) 0 (0%)	10 (52.6%) 1 (100%)	6 (40%) 3 (60%)	9 (60%) 2 (40%)
		p = 1.000 by Fisher		p = 0.617 by Fisher	
TNF-α -1150 T/C	TT TC+CC	8 (44.4%) 1 (50%)	10 (55.6%) 1 (50%)	9 (45%) 0	11 (55%) 0
		p = 1.000 by Fisher		p = -	
TNF-α -1234 C/G	CC CG+GG	3 (30%) 6 (60%)	7 (70%) 4 (40%)	7 (38.9%) 2 (100%)	11 (61.1%) 0 (0%
		p = 0.370 by Fisher		p = 0.189 by Fisher	

Table V. *Correlation of the TNF-a SNPs with clinical manifestations of CanL.*

SNPs	Genotypes	Dermal lesions		Lymph node enlargement		Ocular lesions		Cachexia	
		NO	YES	NO	YES	NO	YES	NO	YES
-40 C/A	C/C (%)	1 (100%)	0 (0%)	1 (100%)	0 (0%)	0 (0%)	1(100%)	0 (0%)	1(100%)
	C/A + A/A(%)	4 (21%)	15(79%)	3(15.8%)	16(84.2%)	5 (26.3%)	14(73.6%)	12(63.1%)	7(36.9%)
		p = 0.250 by Fisher		p = 0.200 by Fisher		p = 1.000 by Fisher		p = 0.400 by Fisher	
- 1 1 3 4 T/G	T/T (%)	4(26.7%)	11(73.3%)	3(20%)	12(80%)	2(13.3%)	13(86.7%)	11 (73.3%)	4(26.7%)
	T/G +G/G(%)	1(20%)	4(80%)	1(20%)	4(80%)	3 (60%)	2(40%)	1(20%)	4(80%)
		p = 1.000 by Fisher		p = 1.000 by Fisher		p = 0.073 by Fisher		p = 0.109 by Fisher	
- 1 1 5 0 T/C	T/T (%)	5(25%)	15(75%)	4(20%)	16(80%)	5(25%)	15(75%)	11(61.1%)	7(38.9%)
	T/C + C/C (%)	0	0	0	0	0	0	0	0
		p = ns		p = ns		p = ns		p = ns	
- 1 2 4 3 C/G	C/C (%)	4(22.2%)	14(77.8%)	3(16.7%)	15(83.3%)	5(27.8%)	13(72.2%)	11(61.1%)	7(38.9%)
	C/G + G/G (%)	1 (50%)	1(50%)	1 (50%)	1(50%)	0(0%)	2(100%)	1 (50%)	1(50%)
		p = 0.447 by Fisher		p = 0.368 by Fisher		p = 1.000 by Fisher		p = 1.000 by Fisher	

both in the cases and controls, thus alleles are not inherited as a block.

DISCUSSION

In CanL the outcome of the infection is highly variable. However, it is widely accepted that there is host genetic control of susceptibility or resistance to CanL.

In the present study, in order to identify genetic factors associated with resistance or susceptibility to CanL, the nucleotide sequence upstream of the ORF

of the canine TNF- α gene was determined for the first time.

TNF-α exhibits a crucial role during the immune response against *Leishmania* parasites. The regulation of TNF-α production at all time points of an inflammatory process is considered a determining factor for the outcome of infection (10, 16).

In CanL studies, applying real-time PCR, similar quantities of TNF-α mRNA were detected in PBMC and in sera of both symptomatic and asymptomatic dogs (8, 17-18). However, a recent study showed that asymptomatic dogs had the highest expression of TNF-α and low parasite burden in lymph nodes compared to symptomatic ones, indicating that this cytokine plays a protective role against infection (19).

The correlation of the SNPs located upstream of the canine TNF-α ORF with susceptibility or resistance to CanL could be due to the fact that these specific SNPs coincide with certain transcription factor binding sites. The *in vivo* production of TNF-α is transcriptionally regulated (20-21). Regarding TNF-α, it has been shown that the SNPs modulating the regulation of transcription are those within the promoter region (21-23).

Our data suggest that the SNP TNF-α -40 C/A, which was associated with susceptibility to CanL, constitutes a binding site of one or more transcription factors with suppressive effect, resulting in reduced gene expression and thus low cytokine levels in various tissues. On the contrary, the SNP TNF-α -1243, which was associated with resistance to CanL constitutes a binding site of one or more transcription factors with enhancing effect, resulting in increased gene expression and thus high cytokine levels in various tissues.

TFSearch analysis, in order to identify transcription factor binding sites within the vicinity of SNPs, predicted binding sites of important transcription factors in these SNPs. However, the function of the specific transcription factors still remains to be determined.

The results of the present study could provide a potential diagnostic tool for detection of animals at risk of CanL. Moreover, the genetic and immunological evidence pointing to TNF-α as a clear risk factor for disease suggests that immunotherapies directed at regulation of its production might prove of value in the treatment of this disease. Further characterization of SNPs of the TNF-α gene, as well as of other candidate genes, may provide important insights into the immunological mechanisms underlying susceptibility to CanL.

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