Research article

PROMOTER POLYMORPHISMS OF THE CANINE SCL11A1 GENE ARE CORRELATED WITH SUSCEPTIBILITY TO CANINE LEISHMANIOSIS

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In enzootic areas the prevalence estimates of canine leishmaniosis are high whereas only a proportion of dogs exhibit the clinical disease, thus implying a role of host genetics. The type of the triggered immune response remains a crucial determining factor for the diverse outcome of this parasitosis. The Solute Carrier Family 11 member 1 (SLC11A1) is a protein, which plays a central role in macrophage function and is implicated in the regulation of the immune response. An extended study with 73 resistant and 75 susceptible to *Leishmania* dogs was conducted. A fragment of the promoter region of the canine SLC11A1 gene was amplified and digested providing the different genotypes for three previously recorded single-nucleotide polymorphisms (SNPs) (SNP1 T151C, SNP2 A180G, SNP3 G318A) for each animal. Statistical analyses revealed that SNP2 A180G in heterozygosity (AG) as well as SNP3 G318A in homozygosity (AA) are correlated with susceptibility to canine leishmaniosis.

Key words: dogs, leishmaniosis, polymorphisms, Solute Carrier Family 11 member 1

INTRODUCTION

Canine leishmaniosis (CanL) is a complex, life threatening disease with a variety of clinical manifestations [1]. Epizootical studies using molecular techniques in enzootic areas have confirmed that CanL has a prevalence involving as much as 67%–80% of the dog population [2,3]. However, this high prevalence estimate is accompanied by a low rate of apparent clinical disease. One determining factor for the polarity of

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clinical manifestations is the balance between humoral and cellular immune responses [1]. Protective immunity and thus resistance against *Leishmania* parasites is mediated by CD4+ Th1 cellular responses, where the cytokines interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α) and interleukin-12 (IL-12) are released inducing macrophage activation and nitric oxide (NO) killing of parasites. On the contrary, the failure to control infection is related to the Th2 immune response, increased activity of humoral immunity and the production of IL-4, IL-5, IL-6, IL-10 and IL-13 [4]. Other genetic factors, such as major histocompatibility complex (MHC) class II and TNF-α have been analyzed for CanL and significant correlations have been reported [5,6].

SLC11A1 (Solute Carrier Family 11 member 1), also known as Nramp1 (Natural resistance associated macrophage protein 1), is a metal ion transporter protein, which is expressed in the lysosomal compartment of professional phagocytes [7]. The SLC11A1 protein is crucial for the initial activation of macrophages and is critical for resistance to intracellular pathogens [8]. Moreover, SLC11A1 is implicated in the regulation of the expression of TNF-α and IL-1b [9] and it enhances the induction of nitric oxide synthase (iNOS) and NO, which are toxic to *Leishmania* parasites [10].

SLC11A1 is encoded by the positionally cloned *Ity/Lsh/Bcg* gene, which was firstly described in genetic studies of mouse models for its role in regulating susceptibility and resistance to *Leishmania*, *Salmonella* and *Mycobacterium* [8,11]. The canine SLC11A1 gene has been located on dog chromosome CFA37 and spans for 9kb, including a promoter region of 700 base pairs (bp) and 15 exons (GenBank accession number AF091049) [12]. Up to date, there is increasing evidence that genetic variation within the SLC11A1 can determine susceptibility or resistance to CanL [13,14]. Three single nucleotide polymorphisms (SNPs) within a region of 491 bp, mapped in the promoter of the canine SLC11A1 gene have been identified by sequence variant analysis conducted by direct sequencing of the cDNA and characterized in susceptible dogs from various breeds, i.e. SNP1 T151C, SNP2 A180G και SNP3 G318A [13,14].

The aim of the study was to corroborate the association of these three sequence variants in the promoter region within the canine SLC11A1 gene with susceptibility or resistance to CanL in an extended case-control cohort.

MATERIAL AND METHODS

Animals and DNA extraction

Infected dogs (n=148) from 14 different breeds were included in the study. All dogs were living in Northern Greece and had owners. From each dog we used the residual of peripheral whole blood, which was taken anyway at routine check ups by practitioners. For all dogs *Leishmania* infection was verified, either by positive cytology following lymph node aspiration and/or by positive anti-*Leishmania* antibody titre. In detail, for the detection of antibodies against *Leishmania* spp. an in-house enzyme-linked immunosorbent assay (ELISA) was used as described previously [15]. Furthermore, it

was confirmed that all dogs were free of symptoms of any other disease with possible immunosuppressive effect and were seronegative (spot ELISA) for *Ehrlichia canis* and *Dirofilaria immitis*. Dogs were divided in two groups: resistant and susceptible. Breeds were equally distributed to both groups so as to exclude breed effect (Table 1). The group of resistant dogs comprised of 73 apparently healthy dogs (39 males and 34 females). All of these dogs were older than 7 years and never exhibited any clinical manifestations of CanL, although examined regularly by a practitioner. The group of susceptible dogs consisted of 75 symptomatic dogs (34 males and 41 females) that exhibited clinically severe disease and/or numerous relapses. Genomic DNA was extracted from 0.3 ml of peripheral whole blood of the examined animals using the Gentra Puregene Blood Kit according to the manufacturer's manual (Qiagen, USA).

Table 1. Breed distribution amongst resistant and susceptible dogs

Breed	Resistant dogs	Susceptible dogs
Alaskan malamute	6	6
Belgian shepherd (Groenendal)	5	5
English setter	5	5
Boxer	4	5
English pointer	3	3
Labrador retriever	2	2
Cocker spaniel	5	5
Doberman pinscher	9	9
German shepherd	8	8
Siberian husky	5	5
Rottweiler	4	5
Collie	3	3
Ghekas (Greek pointer)	11	11
Kurzhaar	3	3
Total	73	75

Animal genotyping

A semi-nested polymerase chain reaction (PCR) was applied for the amplification of a 420 bp fragment of the promoter of the canine SLC11A1 gene. Three primers were designed using the IDT OligoAnalyzer 3.1 software. The primers used in the first assay were SLC11A1-F (sense), 5'-GTGGCAGGATCGGGACCAGA-3' and SLC11A1-R1 (antisense), 5'-CAGCTGATCTCAGCTGTCCTC-3'. Each reaction was performed in a BIORAD Thermal Cycler at a final volume of 25 µl containing 1.5 µl genomic DNA extract, 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 1 x High

Fidelity PCR Buffer with MgCl2 (10x). Cycling conditions consisted of a pre-heat step of 94°C for 7 minutes, followed by 32 cycles of 95°C for 30 sec, 58°C for 30 sec, 72°C for 20 sec and a final step of 72°C for 3 min. The semi-nested PCR was performed using the SLC11A1-F (sense) and the internal primer SLC11A1-R2nest (antisense), 5'-ACACTTTCCAAAGGCAGAAGTGG-3'. Each reaction was performed in a final volume of 25 µl containing 1 µl of the product from the first PCR, 0.2 µM of each primer, 0.2 µM dNTPs, 0.6 U High fidelity PCR enzyme mix (K0191, Fermentas) and 1 x High Fidelity PCR Buffer with MgCl$_2$ (10x). Cycling conditions consisted of a pre-heat step of 94°C for 7 min, followed by 35 cycles of 95°C for 30 sec, 59°C for 30 sec, 72°C for 20 sec and a final step of 72°C for 3 min. All products were electrophorized on 1.5% ethidium bromide stained agarose gels and visualized under UV illumination.

Genotyping by restriction fragment length polymorphism (RFLP) assays

Genotyping analyses in regard to the three SNPs were accomplished by enzymatic digestion of the DNA samples in question with specific restriction endonucleases. In particular, the enzymes Hpy188I, Bsp1286I and AciI (New England Biolabs) were selected using NEBcutter V2.0 (http:/tools.neb.com/nebcutter2) to discriminate between SNP1 T151C, SNP2 A180G and SNP3 G318A, respectively (Table 2). All restriction reactions were done in a final volume of 15 µl containing 6 µl PCR product, 1.5 µl NE buffer (10x), 0.3 µl of the enzyme and 7.2 µl distilled water with incubation at 37 °C for 16h. The restricted fragments were electrophorized on 2.5% ethidium bromide stained agarose gels and visualized under UV illumination.

Table 2. The restriction enzymes for each SNP, their digestion sites and resulting fragments for each allele

| Enzyme	SNP	Digestion site	Alleles	Resulting fragments fragments (bp)
Hpy188I	SNP1 T151C	T151	TT	40,66,150,164
			CT	40,66,106,105,164
			CC	106,105,164
Bsp1286I	SNP2 A180G	G180	AA	89,331
			AG	70,89,260,331
			GG	70,89,260
AciI	SNP3 G318A	G318	GG	34,179,206
			GA	34,179,206,385
			AA	34,385

Statistical analysis

Analyses were carried out with Statistical Package for the Social Sciences (SPSS, version 15.0, SPSS Inc., Illinois, USA). GenePop version 4.0.10 (http://genepop.curtin.edu.au/) was used for calculating genotypic as well as allelic frequency distributions and the deviations from Hardy-Weinberg equilibrium.

Informed consent: Informed consent for the use of the residual 0.3 ml blood and every dog's medical record in the form of written permission has been obtained from the owners for all dogs included in the study.

RESULTS

For SNP1 T151C only the TT genotype was detected in all dogs (Figure 1) and thus a chi-square test was not conducted. For SNP2 A180G both genotypes (AA and AG) were detected in both groups (Figure 2). The AA genotype was detected in 70 resistant and 51 susceptible dogs. The AG genotype was detected in 3 resistant and 24 susceptible dogs. SNP2 A180G was statistically correlated with susceptibility to CanL ($p < 0.05$) (Table 3). For SNP3 G318A both genotypes (GA and AA) were detected in both groups (Figure 3). The GA genotype was detected in 71 resistant and 57 susceptible dogs. The AA genotype was detected in 2 resistant and 18 susceptible dogs. Subsequent statistical analysis demonstrated that SNP3 G318A might be a susceptibility factor since the presence of G318A polymorphism in homozygous state (AA) was remarkably higher ($p < 0.083$) in susceptible dogs (Table 4). Finally, according to the statistical analyses all loci were in Hardy–Weinberg equilibrium for both populations ($p > 0.05$).
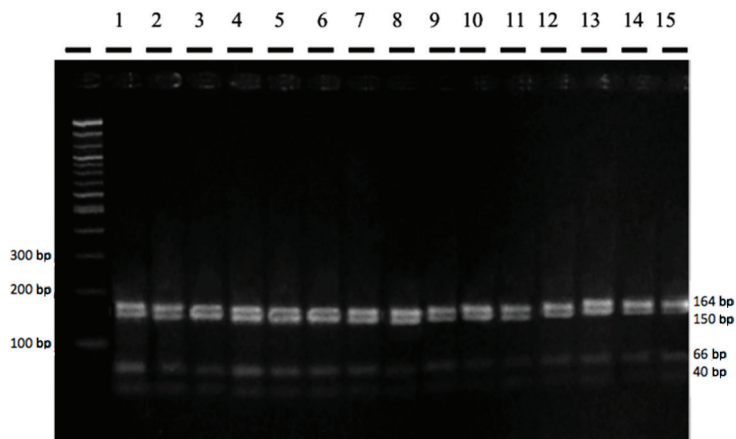
Figure 1. RFLP products after digestion with Hpy188I analyzed by electrophoresis on 2.5% agarose gels, stained with ethidium bromide and visualized on a UV transilluminator. L: DNA ladder and 1 - 15: RFLP products of fifteen dogs with TT genotype (40,66,150 and 164 bp fragments).
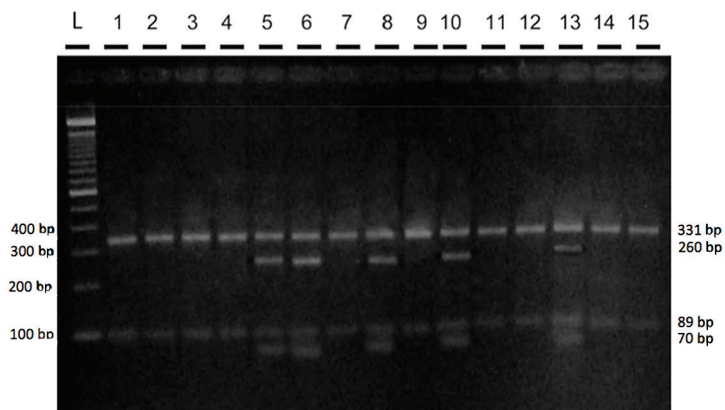
Figure 2. RFLP products after digestion with Bsp1286I analyzed by electrophoresis on 2.5% agarose gels, stained with ethidium bromide and visualized on a UV transilluminator. L: DNA ladder, 1-4,7,9,11,12,14,15: RFLP products of ten dogs with AA genotype (89 and 331 bp fragments) and 5,6,8,10,13: RFLP products of five dogs with AG genotype (70,89,260 and 331 bp fragments).
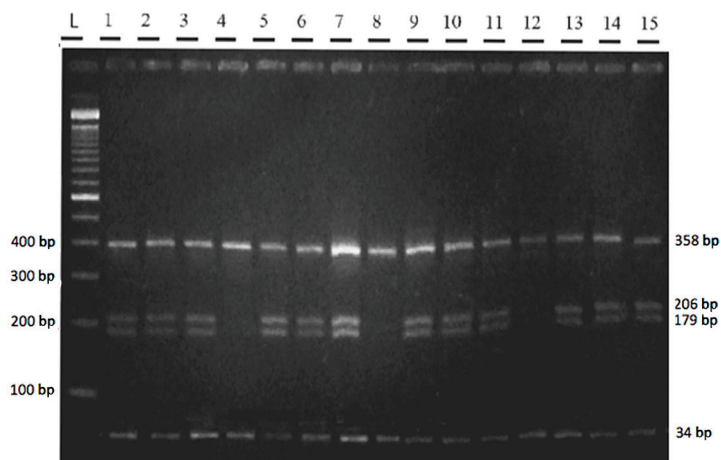
Figure 3. RFLP products after digestion with AciI analyzed by electrophoresis on 2.5% agarose gels, stained with ethidium bromide and visualized on a UV transilluminator. L: DNA ladder, 1-3, 5-7, 9-11, 13-15: RFLP products of fourteen dogs with GA genotype (34,179,206 and 385 bp fragments) and 4,8,12: RFLP products of three dogs with AA genotype (34 and 385 bp fragments).

Table 3. Genotype distribution and allele frequencies of SLC11A1-SNP2 A180G in resistant and susceptible dogs

Scl11α1-SNP2 A180G	Resistant dogs	Susceptible dogs
genotype	n (%)	n (%)
AA	70 (95.9)	51 (68)
AG + GG	3 (4.1)	24 (32)
p = 0.000 Pearson (ǫ)		
Total	73 (100)	75 (100)
allele		
A	143 (0.979)	126 (0.840)
G	3 (0.021)	24 (0.160)
Total (frequency)	146 (1)	150 (1)
p = 0.0001		

Table 4. Genotype distribution and allele frequencies of SLC11A1-SNP3 G318A in resistant and susceptible dogs

Scl11α1-SNP3 G318A	Resistant dogs	Susceptible dogs
genotype	n (%)	n (%)
AA	2 (2.7)	18 (24)
GA + GG	71 (97.3)	57 (76)
Total	73 (100)	75 (100)
p = –		
allele		
G	71 (0.486)	57 (0.380)
A	75 (0.514)	93 (0.620)
Total (frequency)	146 (1)	150 (1)
p = 0.083		

DISCUSSION

In CanL some dogs are resistant and thus remain asymptomatic, while others are susceptible and develop the clinical form of the disease [1,2]. A number of genetic loci have been involved in susceptibility or resistance, although the mechanisms are not yet thoroughly clarified [3]. In a cohort of Brazilian mongrel dogs naturally exposed to *L. infantum* the presence of MHC class II allele DLA-DRB1*01502 increased the risk of infection [5]. A correlation of two SNPs, i.e. TNF-α -40 C/A and TNF-α -1243 C/G, located upstream the canine TNF-α open reading frame with susceptibility or resistance to CanL, respectively, was determined from a study of naturally infected dogs in Greece [6].

SLC11A1 is highly conserved among humans, mice and dogs [12] and it is implicated in susceptibility to *L. infantum* infection in mice [8,16]. In humans one repeated GT promoter sequence and 823C/T in exon 8 have been associated with susceptibility to visceral leishmaniosis [17-19], while SNPs in the 3'UTR region, in exon 8 and the D543N variant have been correlated to cutaneous leishmaniosis [20,21].

In the present study three previously described SNPs (SNP1 T151C, SNP2 A180G και SNP3 G318A) in the promoter of the canine SLC11A1 gene were further analyzed in a case-control study of 148 dogs. Statistical analyses revealed a significant association either for the SNP2 A180G in heterozygosity (AG) or for the SNP3 G318A in homozygosity (AA) with susceptibility to CanL while in other studies all three of these SNPs were implicated in the control of infection [13,14,22].

SLC11A1 is implicated in Th0 cell differentiation of infected animals. Studies have shown that congenic mice carrying the wild-type SLC11A1 allele mounted a predominantly Th1 immune response to vaccination and resolved lesions upon challenge with *Leishmania major* whereas mice carrying the mutant allele mounted a Th2 immune response and lesions expanded following challenge [23]. The mechanism of this action is not clarified, although it has been proven that the ability of SLC11A1 to induce the pathways of IL-10 and IL-12 in macrophages and dendritic cells (DCs) is involved [24]. The balance between these cytokines reflects the development of the Th1 cellular immune response [25]. It has been proven that the ratio of IL-10 production to IL-12 is higher in DCs from mutant mice than in those from wild-type mice [26].

SLC11A1 influences innate immunity against *Leishmania* parasites by interfering directly with antigen processing [20,27], probably by regulating the activity of proteases in the endosomal compartment [23], which is dependent on metal ions [28]. It has been proven that macrophages of congenic mice carrying the wild type of SLC11A1 have impaired iron homeostasis and deficient antigen processing compared to the ones bearing the polymorphism [27]. SLC11A1 also influences antigen processing by regulation of MHC class II molecules [29]. DCs of mice carrying the wild-type allele show increased expression of MHC class II than DCs from mutants [26].

In dogs silico analysis has revealed that within the vicinity of these SNPs are putative transcription factor binding sites [14]. This fact affects the binding ability of transcription factors and results in changes of transcriptional machinery [30]. One SNP of the human SLC11A1 gene, which was associated with infectious diseases, encodes four alleles, which differ in their ability to drive gene expression [17]. This may explain the diverse expression of SLC11A1 gene in dogs bearing the polymorphic sites [12].

In conclusion, in the present study the presence of SNP2 A180G in heterozygosity (AG) and the presence of SNP3 G318A in homozygosity (AA) were correlated with susceptibility to CanL. Future studies of more SNPs in candidate genes are required so as to clarify the roles of different polymorphic variants in determining CanL severity.

Authors' contributions

SI participated in the design of the study, carried out the molecular genetic studies and wrote the manuscrip. PS and KA helped to carry out the molecular genetic studies and performed the statistical analyses. PE participated in the design of the study and revised the manuscript. DCI helped to carry out the molecular genetic studies and revise the manuscript. HM and FS conceived the study, participated in its coordination and revised the manuscript. All authors read and approved the final version to be published.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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PROMOTERI POLIMORFIZAMA SCL11A1 GENA PASA SU U KORELACIJI S PRIJEMČIVOŠĆU NA LAJŠMANIOZU PASA

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U regionima gde je prevalencija lajšmanioze pasa visoka, retko se nalaze klinički slučajevi oboljenja što ukazuje na ulogu genetskih faktora kod ove bolesti. Tip imunskog odgovora koji je izazvan, predstavlja bitan faktor koji određuje različite ishode u slučaju ove parazitoze. Član 1 *Solute Carrier Family 11* (SLC11A1) je protein, koji igra centralnu ulogu u funkcionisanju makrofaga pri čemu je uključen u regulaciju imunskog odgovora. Obavljena je obimna studija koja je uključivala 73 rezistentna i 75 osetljivih pasa na Leishmania infekciju. Segment promoter regiona SLC11A1 gena kod pasa je amplifikovan, obavljena je njegova digestija pri čemu su dobijeni različiti genotipovi za tri prethodno uočena polimorfizma jednog nukleotida (SNP) (SNP1 T15C, SNP2 A180G, SNP3 G318A), za svaku životinju. Statistička analiza je ukazala da su SNP2 A180G u heterozigotnosti (AG) kao i SNP3 G318A u homozigotnosti (AA) u korelaciji sa osetljivošću odnosno, prijemčivošću na lajšmaniozu pasa.