Journal of Heredity 2007:98(5):518–525
doi:10.1093/jhered/esm048
Advance Access publication July 4, 2007

Analysis of Candidate Susceptibility Genes in Canine Diabetes

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Abstract

Canine diabetes is a complex genetic disease of unknown aetiology. It affects 0.005–1.5% of the canine population and shows a clear breed predisposition with the Samoyed being at high risk and the Boxer being at low risk of developing the disease. Canine diabetes is considered to be a disease homologue for human type 1 diabetes (T1D). It results in insulin deficiency as a consequence of autoimmune destruction of islet β-cells in the pancreas and is believed to be mediated by Th1 cytokines (*IFNγ, TNFα,* and *IL-2*). A number of genes have been associated with type 1 diabetes in humans, including the human leukocyte antigen region, the insulin variable number tandem repeat, *PTPN22, CTLA4, IL-4,* and *IL-13*. As yet, these genes have not been evaluated in canine diabetes. In this study, 483 cases of canine diabetes and 869 controls of known breed were analyzed for association with *IFNγ, IGF2, IL-10, IL-12β, IL-6, insulin, PTPN22, RANTES, IL-4, IL-1α* and *TNFα*. Minor allele frequencies were determined for these genes in each breed. These data were used for comparative analyses in a case–control study, and clear associations with diabetes were identified in some breeds with certain alleles of candidate genes. Some associations were with increased susceptibility to the disease (*IFNγ, IL-10, IL-12β, IL-6, insulin, PTPN22, IL-4,* and *TNFα*), whereas others were protective (*IL-4, PTPN22, IL-6, insulin, IGF2, TNFα*). This study demonstrates that a number of the candidate genes previously associated with human T1D also appear to be associated with canine diabetes and identifies an *IL-10* haplotype which is associated with diabetes in the Cavalier King Charles Spaniel. This suggests that canine diabetes is an excellent comparative and spontaneously occurring disease model of human T1D.

Type 1 diabetes (T1D) is an autoimmune disease that develops as a consequence of autoimmune β-cell destruction in the pancreas. It accounts for 10–15% of the human diabetic population and is usually diagnosed in patients under the age of 30. It is a complex genetic disease, and its development is due to interactions between genetic predisposition and environmental exposures.

Canine diabetes mellitus (DM) is a common endocrine disease having a prevalence of 0.005–1.5% (Mattheeuws et al. 1984). It shares many similarities with human T1D (Davison et al. 2003, 2005) although it is commonly diagnosed in older dogs (7–12 years). Similarities include the lack of increased blood glucose levels after the β-cell stimulation with glucose or glucagon, the progressive destruction of β-cells, (indicative from the increasing level of C-peptide concentrations after insulin treatment), and the presence of antibodies against islet components (GAD65, IA-2, insulin) in humans and against β-cells in newly diagnosed dogs. The target of the canine diabetic antibodies and their clinical relevance has not been identified to date (Davison et al. 2003). There are 2 main differences between canine DM and human T1D in that the autoantibodies produced in dogs with DM do not target insulin, as is seen in humans (Hoenig 2002), and postmortem canine islets do not show the lymphocytic infiltration, commonly seen in humans. It is possible, however, that lymphocyte infiltration occurs early in the canine form of the disease and has dissipated before the death of the animal.

The genetic contribution to human T1D has been extensively studied and a genetic hierarchy established in which the human leukocyte antigen (HLA) region contributes approximately 50% of the genetic predisposition to T1D followed by insulin, *PTPN22* (Bottini et al. 2004; Qu et al. 2005), and *CTLA4* (Lee et al. 2001; Turpeinen et al. 2003; Zalloua et al. 2004; Steck et al. 2005). Other candidate genes associated with human T1D include *IL-4* and the *IL-4 receptor* (Berman et al. 1996; Zaccone et al. 1999), *IL-10, IL-13, IL-2,* and *TGFβ*

(Zaccone et al. 1999; Kretowski et al. 2002; Ide et al. 2004; Mojtahedi et al. 2006). There are also a number of potential environmental factors including food proteins (Lefebvre et al. 2006), intestinal permeability (Watts et al. 2005), and viral exposure (Honeyman et al. 2000; Rewers 2001), which are believed to be functional in the cause of the disease.

There is some uncertainty as to whether the canine DM represents T1D or latent autoimmune diabetes of adults (LADA), an autoimmune form of diabetes characterized in humans by onset after 30 years of age and an insulin-free period after initial diagnosis. Canine diabetes is present in dogs aged between 7 and 12 years and requires insulin from diagnosis. The age of onset in dogs is similar to that of LADA patients, but the requirement of insulin from diagnosis is characteristic of general T1D.

Few candidate gene studies have been carried out using LADA cohorts; however, the HLA region (Turner et al. 1997; Tuomi et al. 1999), the insulin variable number tandem repeat (VNTR) (Cerrone et al. 2004; Matejkova-Behanova et al. 2004), *CTLA4* (Caputo et al. 2005), and *TNF-2* (Vatay et al. 2002) have been identified as risk factors for humans.

Canine DM also shares some similarities with monogenic forms of human diabetes (maturity onset diabetes of the young [MODY]), of which there are 6 types, MODY1–6, caused by defective genes for hepatic nuclear transcription factor 1 (*HNF1*) (Yamagata, Oda, et al. 1996), *HNF4α* (Yamagata, Furuta, et al. 1996), *glucokinase* (Froguel et al. 1992), Insulin promoter factor (*IPF-1*) (Stoffers et al. 1997), *HNF1β* (Horikawa et al. 1997), and *NeuroD1* (Malecki et al. 1999). There are no reported studies on these types of diabetes in the dog to date.

In human T1D, there is a well-documented association between the major histocompatibility complex (MHC) and susceptibility to T1D (Wassmuth and Lernmark 1989; Thorsby and Ronningen 1993; Ionescu-Tirgoviste et al. 2001; Ronningen et al. 2001; Jones et al. 2006), and a similar association has been identified in canine populations. The dog leukocyte antigen (DLA, the canine equivalent of the MHC) shows overrepresentation of 3 haplotypes in the diabetic population (DLA-DRB1*009/DQA1*001/DQB1*008, DLA-DRB1*015/DQA1*006/DQB1*023, and DLA-DRB1*002/DQA1*009/DQB1*001) and underrepresentation of 1 haplotype (DLA-DQ DQA1*004/DQB1*013) (Kennedy et al. 2006).

As with human populations, dog breeds exhibit different susceptibility to diabetes. Some breeds are reported to be highly susceptible to the disease (Samoyed and Cairn Terrier), whereas others appear highly resistant (Boxer and German Shepherd Dog) (Marmor et al. 1982). Analysis of the genetic differences between these breeds could help provide a greater insight into the population variation of diabetes. In addition, the breed variation in susceptibility could serve as an important genetic indicator for identifying susceptibility and resistance genes for the disease. Breeds showing a high prevalence of diabetes would be most suited for the identification of high-risk alleles and haplotypes and those with a low disease prevalence for the identification of protective alleles or haplotypes. With a large number of breeds falling between the 2 extremes, it may be possible to identify a genetic hierarchy and determine which alleles, haplotypes, or genes predominate in disease susceptibility. Identification of such a hierarchy could be indicative of the relationship between immune and environmental components.

We have carried out a single-nucleotide polymorphism (SNP)–based analysis of canine DM in a diabetic cohort containing 483 cases and 869 controls from 30 different breeds for a range of candidate genes which included *IFNγ, IGF2, IL-10, IL-12β, IL-6, insulin, PTPN22, RANTES, IL-4, IL-1α,* and *TNFα*.

Materials and Methods

DNA

DNA was extracted from residual pathology ethylenediaminetetraacetic acid blood samples from 894 dogs from 33 breeds (Table 1) using a standard phenol:chloroform method. These were normalized to 50 ng/μl. DNA samples were whole-genome amplified (WGA) using the GenomiPhi kit (Amersham, Little Chalfont, UK) as sample DNA was limited. WGA was performed as described elsewhere (Short 2006). WGA DNA concentration was measured using PicoGreen. The average yield per sample was 3 μg. All samples were diluted to a final concentration of 5 ng/μl.

SNP Selection

Ninety-three SNPs were chosen from 11 different candidate genes. All SNPs used were novel and were identified from our own SNP discovery investigation: they are listed elsewhere (Short 2006). SNPs were chosen if they encoded a nonsynonymous amino acid change, were located in exonic regions, or were in the region 1.5 kbp upstream of exon 1. The genes analyzed were *IFNγ* (5 SNPs), *IGF2* (5 SNPs), *IL-10* (14 SNPs), *IL-12β* (11 SNPs), *IL-6* (11 SNPs), *insulin* (6 SNPs), *PTPN22* (11 SNPs), *RANTES* (4 SNPs), *IL-4* (8 SNPs), *IL-1α* (6 SNPs), and *TNFα* (12 SNPs) (Table 2).

Taqman Genotyping

Regions of 400-bp sequence with the SNP central were submitted to ABI for the SNP V-scale nonhuman Assay by Design service. Assays were initially tested in a 5-μl volume on a panel of reference canine DNA samples. Test reactions contained 2.5 μl 2× master mix w/o UNG (Eurogentec, Southampton, UK), 0.125 μl 40× assay mix, 0.375 μl Milli-Q water, and 2 μl DNA at 5 ng/μl. Standard amplification conditions were used on a PTC-225 MJ tetrad thermal cycler, 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. Samples were stored at 4 °C in the dark until they could be read on the ABI Prism 7700 Sequence Detector. Fourteen SNPs were genotyped using Taqman, these were 4 SNPs from *RANTES* (2 intronic and 2 exonic), 5 SNPs from *IFNγ* (3 from the promoter region and 2 from exon 4), 2 nonsynonymous coding SNPs from *IL-10*, 2 nonsynonymous coding SNPs from *IL-12β*, and 1 nonsynonymous coding SNP from *IL-6*.

Table 1. Number of controls and cases for each breed

Breed	Controls (*n*)	Cases (*n*)
Labrador Retriever	97	67
German Shepherd Dog	65	1
Retriever	64	7
Boxer	51	0
Jack Russell Terrier	50	33
Yorkshire Terrier	48	23
All Spaniel	39	9
Springer Spaniel	29	9
English Springer Spaniel	5	0
Welsh Springer Spaniel	3	0
Spaniel	2	0
West Highland White Terrier	38	43
Border Collie	36	31
Cocker Spaniel	34	18
All Dachshund	28	15
Miniature Dachshund	1	8
Dachshund	6	7
Short-haired Dachshund	5	0
Long-haired Dachshund	3	0
Wire-haired Dachshund	2	0
Miniature short-haired Dachshund	11	0
English Setter	27	4
Cavalier King Charles Spaniel	25	20
Crossbreed	34	99
Bichon Frise	21	14
All Poodle	20	8
Miniature Poodle	5	5
Toy Poodle	5	2
Poodle	4	1
Standard Poodle	6	0
All Schnauzer	19	10
Miniature Schnauzer	13	10
Giant Schnauzer	4	0
Schnauzer	2	0
Staffordshire Bull Terrier	19	2
Beagle	17	7
Rottweiler	17	5
Bullmastiff	16	1
Doberman	15	7
Border Terrier	14	10
Basset Hound	12	0
Collie	11	11
Great Dane	11	1
Cairn Terrier	11	17
Weimaraner	11	1
Bernese Mountain Dog	10	0
Samoyed	9	20
Total	869	483

Table 2. Distribution of candidate gene SNPs analyzed

Gene	No. SNPs genotyped	Canine chromosome
PTPN22	11	17
IL-10	14	7
IL-12β	11	4
IL-6	11	14
IL-4	8	11
RANTES	4	9
IFNγ	5	10
Insulin	6	18
IL-1α	6	17
TNFα	12	12
IGF2	5	18
Total	93	9 Chromosomes

Sequenom Genotyping

Primers and probes were designed using Sequenom Assay Design software Version 3 and synthesized by Metabion (Germany). Primers were diluted to 100 μM, and plexes pooled to contain 500 nm of each forward and reverse primer. Probes were diluted to 400 μM, and probe pools were split into 50% high-mass and 50% low-mass probes. Probe pools contained 26 μl of each low-mass probe and 52 μl of each high-mass probe in a final volume of 1.5 ml.

Polymerase chain reactions (PCRs) contained 15 ng DNA plated into a 384-well plate and dried down at room temperature overnight. PCR was performed in 5-μl volumes on a PTC-225 MJ tetrad cycler (384 well). Reactions contained 1.25× HotStarTaq PCR buffer, 1.625 mM $MgCl_2$, 500 μM of each deoxynucleoside triphosphate (dNTP), 0.5 U of HotStarTaq, and 100 nm primer pool and were amplified as follows: 95 °C for 15 min; 35 cycles of 95 °C for 20 s, 56 °C for 30 s, 72 °C for 1 min; 72 °C for 3 min. After PCR, reactions were treated with 0.3 U shrimp alkaline phosphatase to inactivate remaining dNTPs. Reactions were incubated at 37 °C for 20 min and denatured at 80 °C for 5 min. iPLEX primer extension was carried out on a Dyad PCR engine. Reactions contained 0.22× iPLEX buffer, 1× iPLEX termination mix, 0.625 μm low-mass primer, 1.25 μm high-mass primer, and 1× iPLEX enzyme and were amplified as follows: 94 °C for 30 s, 40 cycles of 94 °C for 5 s, 5 cycles of 52 °C for 5 s, 80 °C for 5 s, and a final extension of 72 °C for 3 min. Samples were diluted with 25 μl water and desalted using 6 mg resin before being centrifuged for 5 min at 4000 rpm in a Jouan CR4 centrifuge and spotted onto a SpectroCHIP using a Sequenom mass array nanodispenser (Samsung). The 2 nonsynonymous coding SNPs from both *IL-10* and *IL-12β* were genotyped by Sequenom and Taqman to serve as genotyping controls between the 2 platforms and had concordance rates of 97% (data not shown).

Data Analysis

Phenotype data were imported into BCgene (www.bcplatforms.com), and genotype data from the ABI 7700 and Sequenom uploaded directly. Subsets were generated for pedigree breed phenotypes, and the genotype data linked to these. BCgene was used to calculate call rates and Hardy–Weinberg equilibrium (HWE) for controls and cases by breed. SNPs in which the call rate was consistently below 70% and/or the control population was out of HWE were excluded from the analyses.

Minor allele frequencies (MAF) were compared between cases and controls using the BCgene fast association analysis

Table 3. Breed risk categories for diabetes

Breed	Risk[a]
Low	
Boxer	0.07
Weimaraner	0.10
German Shepherd Dog	0.15
Staffordshire Bull Terrier	0.15
Golden Retriever	0.19
Springer Spaniel	0.41
Neutral	
Cocker Spaniel	0.75
Crossbreed	0.80
Labrador Retriever	0.96
Doberman	1.22
Jack Russell Terrier	1.24
Cavalier King Charles Spaniel	1.45
West Highland White Terrier	1.69
Rottweiler	1.74
Moderate	
Poodle	2.40
Border Terrier	2.49
English Setter	2.67
Dachshund	2.83
Collie	2.88
Border Collie	2.88
Schnauzer	3.19
Yorkshire Terrier	3.47
Bichon Frise	3.59
High	
Cairn Terrier	6.80
Samoyed	17.3

High-risk relative risk (RR) >5, moderate risk RR 5 to <2, neutral risk RR 2 to <0.7, low-risk RR <0.7.

[a] Data courtesy of Pet Protect Insurance (46 592 dogs).

tool. Chi square (χ^2), *P* values, odds ratios (ORs), and confidence intervals (CIs) were calculated for each SNP, stratified by breed. SNPs in which one population was monomorphic (cases or controls) were analyzed using a web-based tool (http://www.hutchon.net/confidORnulhypo.htm). Significant *P* values were corrected via permutation testing (*T*1 statistic, CLUMP) (Sham and Curtis, 1995).

Haplotype frequencies and linkage disequilibrium (LD) were estimated using Helix Tree version 4.1.0. Haplotype frequencies were compared between controls and cases using CLUMP. Chi-square values, ORs and CIs were calculated using web-based packages (http://www.georgetown.edu/faculty/ballc/webtools/web_chi.html, http://www.hutchon.net/ConfidOR.htm).

Results

One SNP was removed from the analysis because of a consistently low call rate in both cases and controls (IL-10 14R553), and 12.2% of the SNPs were excluded from the analysis due to noncompliance with HWE in the control population. Exclusions due to noncompliance with HWE were variable across the breeds and were breed/SNP specific. MAF were found to be variable across control populations, and the minor allele in one breed was sometimes found to be the major allele in another, for example, the IL-10 10S308 G allele had a MAF of 0.06 in the Labrador ($n = 97$) but 0.81 in the Golden Retriever ($n = 64$).

A case–control comparison of MAF revealed 13 "protective" allele associations (Table 4) and 24 "susceptibility" allele associations (Table 5). These achieved statistical significance, which was maintained after multiple permutation testing.

Of the protective alleles (Table 4), allele T from SNP IL-6 6K372, allele C from IL-4 13S97, and allele G from IL-4 8R458 were identified in 2 breeds each. The T allele in SNP IL-6 6K372 was identified as being protective in the Jack Russell Terrier and the Border Terrier giving OR of 0.39 (95% CI 0.16–0.93) and 0.21 (0.05–0.88), respectively. The IL-4 13S97 C allele and IL-4 8R458 G allele were protective in the Collie and Cairn Terrier giving OR of 0.14 (0.03–0.78) and 0.06 (0.007–0.48) for 13S97 and 0.14 (0.03–0.08) and 0.06 (0.01–0.56) for 8R458. All other alleles were protective in a single breed.

Table 4. Significant protective SNP associations

Breed	SNP	Minor allele	χ2	*P*	*T1P*	OR	95% CI	Case (2*n*)	MAF case	Control (2*n*)	MAF control
Collie	IL-4 13S97	C	5.79	0.016	0.039	0.14	0.03–0.78	16	0.4	22	0.5
Collie	IL-4 8R458	G	5.63	0.018	0.029	0.14	0.03–0.80	16	0.13	20	0.5
Crossbreed	PTPN22_15	T	5.66	0.017	0.031	0.21	0.05–0.85	162	0	72	0.03
Dachshund	**IL-6 6R431**	**G**	**19.24**	**0.000**	**0.001**	**0.06**	**0.03–0.16**	**28**	**0**	**48**	**0.48**
Labrador	INS 8	G	4.79	0.028	0.019	0.05	0.22–0.93	96	0.11	156	0.22
Cocker Spaniel	**INS1**	**C**	**6.73**	**0.009**	**0.020**	**0.11**	**0.03–0.37**	**28**	**0**	**58**	**0.2**
Border Terrier	**IGF2 10**	**A**	**8.58**	**0.003**	**0.007**	**0.11**	**0.03–0.49**	**16**	**0**	**22**	**0.38**
Border Terrier	IL-6 6K372	T	4.99	0.025	0.031	0.21	0.05–0.88	20	0.15	26	0.46
Cairn Terrier	**IL-4 13S97**	**C**	**7.18**	**0.007**	**0.018**	**0.06**	**0.01–0.48**	**26**	**1**	**16**	**0.25**
Cairn Terrier	IL-4 8R458	G	8.83	0.003	0.015	0.06	0.01–0.56	26	0.04	12	0.42
Cairn Terrier	**TNF 9585**	**C**	**8.15**	**0.004**	**0.009**	**0.07**	**0.01–0.44**	**26**	**0**	**18**	**0.28**
Jack Russell Terrier	IL-6 6K372	T	4.61	0.032	0.035	0.39	0.16–0.93	36	0.25	78	0.46
West Highland White Terrier	TNF 9367	C	5.77	0.016	0.026	0.35	0.15–0.84	64	0.14	66	0.32

T1P = *P* value after permutation testing, cases and controls are 2*n* as alleles are being compared, breeds in bold had one monomorphic population.

Table 5. Significant susceptibility SNP associations

Breed	SNP	Minor allele	$\chi 2$	*P*	*T1P*	OR	95% CI	Case (2*n*)	MAF case	Control (2*n*)	MAF control
Collie	**IL-4 25Y336**	**C**	**8.37**	**0.004**	**0.004**	**15.85**	**2.40–106.0**	**14**	**0.25**	**20**	**0**
Dachshund	IL-12b 2M407	A	5.18	0.023	0.030	3.20	1.16–8.85	26	0.62	42	0.33
Dachshund	IL-12b 3R196	G	4.48	0.034	0.042	2.97	1.07–8.26	26	0.62	40	0.35
Poodle	PTPN22_3	G	6.15	0.013	0.017	5.23	1.34–20.45	14	0.71	34	0.31
Schnauzer	IL-4 1K110	T	8.18	0.004	0.005	14.38	1.64–126.08	12	0.92	30	0.43
Schnauzer	IL-4 2M351	C	6.06	0.014	0.025	6.80	1.31–35.41	14	0.86	32	0.47
Cavalier King Charles Spaniel	IL-10 11R124	A	5.17	0.023	0.040	3.30	1.16–9.38	34	0.65	28	0.36
Cavalier King Charles Spaniel	IL-10 13Y85	T	7.07	0.008	0.013	3.85	1.40–10.59	38	0.66	30	0.33
Cavalier King Charles Spaniel	IL-10 14R553	G	5.37	0.020	0.038	4.05	1.2–13.54	24	0.63	24	0.29
Cavalier King Charles Spaniel	IL-10 1R105	A	5.78	0.016	0.026	3.33	1.23–9.03	36	0.67	32	0.38
Cavalier King Charles Spaniel	IL-10 1R117	A	5.17	0.023	0.040	3.30	1.16–9.38	34	0.65	28	0.36
Cavalier King Charles Spaniel	IL-10 1R218	G	5.17	0.023	0.040	3.30	1.16–9.38	34	0.65	28	0.36
Cavalier King Charles Spaniel	IL-10 2R420	G	6.29	0.012	0.024	3.76	1.31–10.81	34	0.68	28	0.36
Cavalier King Charles Spaniel	IL-10 6Y135	C	7.28	0.007	0.013	4.00	1.43–11.18	36	0.67	30	0.33
Cocker Spaniel	IL-6 20R191	G	14.7	0.000	0.001	8.72	2.68–28.35	26	0.81	40	0.33
Cocker Spaniel	IL-6 6R431	G	4.84	0.028	0.040	2.81	1.10–7.17	34	0.47	50	0.24
Cocker Spaniel	TNF 10513	A	6.94	0.008	0.016	4.01	1.38–11.66	32	0.41	48	0.15
Border Terrier	IL-12b 1Y90	T	5.17	0.023	0.023	9.62	1.01–91.16	18	0.5	26	0.33
Jack Russell Terrier	IFNγ 5M532	C	4.44	0.035	0.036	2.54	1.05–6.11	34	0.25	74	0.17
Jack Russell Terrier	INS 8	G	7.87	0.005	0.012	4.31	1.48–12.52	28	0	70	0.05
West Highland White Terrier	IL-6 6K372	T	7.96	0.005	0.010	7.07	1.52–32.94	68	0.18	68	0.03
West Highland White Terrier	TNF 10513	A	6.46	0.011	0.010	6.15	1.29–29.33	62	0.16	66	0.03
Yorkshire Terrier	IL-6 20R191	G	7.09	0.008	0.013	3.97	1.38–11.39	44	0.34	52	0.12
Yorkshire Terrier	IL-6 20R240	A	5.67	0.017	0.029	2.85	1.18–6.91	56	0.27	88	0.11

T1P = *P* value after permutation testing, cases and controls are 2*n* as alleles are being compared, breeds in bold had one monomorphic population.

Of the high-risk alleles (Table 5), the most striking observation was the number of *IL-10* alleles showing association to diabetes in the Cavalier King Charles Spaniel. This breed showed 8 positive associations for this gene, all of which had OR between 3.30 and 4.05. Such a high number of associated alleles in this gene were indicative of a risk haplotype for this breed. *IL-10* did not show any associations for any other breed in this analysis.

The IL-6 20R191 G allele appeared as a risk factor in the Cocker Spaniel (OR 8.72, CI 2.68–28.35) and the Yorkshire Terrier (OR 3.97, CI 1.38–11.39); but all other risk alleles were identified in a single breed only.

Four breeds showed increased risk of diabetes with 2 alleles from the same gene. The Dachshund had 2 risk alleles in IL-12β, the A allele in 2M407 and the G allele in 3R196; the Schnauzer had 2 risk alleles for *IL-4*, the T allele in 1K110, and the C allele in 2M351; the Cocker Spaniel and Yorkshire Terrier had 2 risk alleles for *IL-6*, the G allele in 20R191 was a risk factor for both breeds, whereas the G allele in 6R431 was a risk factor for the Cocker Spaniel and the A allele in 20R240 was a risk factor for the Yorkshire Terrier.

Three alleles were identified as being protective in 1 breed while increasing the risk of diabetes in another. These were the G allele in IL-6 6R431 which was protective in the Dachshund (OR 0.06, CI 0.03–0.16) while increasing susceptibility to diabetes in the Cocker Spaniel (OR 2.81, CI 1.1–7.17), the G allele in INS8, protective in the Labrador (OR 0.05, CI 0.22–0.93) while increasing the risk of diabetes in the Jack Russell Terrier (OR 4.31, CI 1.48–12.52), and the T allele in IL-6 6K372 which is protective in the Border Terrier (OR 0.21, CI 0.05–0.88) but increases the risk of diabetes in the West Highland White Terrier (OR 7.07, CI 1.52–32.94).

CIs obtained for some associations were large (IL-4 25Y336 in the Collie, CI 2.4–106; IL-4 1K110 in the Schnauzer, CI 1.64–126.08; IL-12β 1Y90 in the Border Terrier, CI 1.1–91.6), due to the small sample numbers.

Haplotype analysis of the *IL-10* gene in the Cavalier King Charles Spaniel identified 2 haplotypes, haplotype 1 (**CAGGA**T**A**AC**T**AGC**G**) was found in 63% of the controls and 34% of the diabetics, whereas haplotype 2 (**TGAAG**T**G**AC**C**AGC**A**) was found in 37% of the controls and 66% of the diabetics. A case–control comparison of these haplotype frequencies gave a χ^2 of 7.48, $P = 0.005$ (0.006 after permutation testing), and an OR of 3.25 (95% CI 1.36–7.75). These haplotypes contained 6 markers that were monomorphic in this breed (1K362, 3M171, 4Y100, 6R426, 9R210, 10S308) with polymorphic markers, in bold (13Y85, 14R553, 1R105, 1R117, 1R218, 2R420, 6Y135, 11R124) having R^2 values of 0.999.

Discussion

We identified 37 SNP allele associations with canine diabetes, of which 13 were protective and 24 increased susceptibility to the disease. Individual SNP associations identified with canine DM were variable between breeds with various markers

being protective in some breeds while increasing the risk of developing the disease in others. It would be expected that protective alleles are categorically protective and that risk alleles categorically infer risk. However, this was not found in this study. Plausible explanations could be that the number of samples in each breed group is small (as reflected in some of the confidence intervals observed) and that a greater level of uncertainty exists with these observations. It is also possible that some associations could represent false positives, whereas others are real or that the identified, associative marker is in LD with both a causative and a protective allele causing ambiguous associations for the alleles in question. However, it is more likely that canine DM has a different aetiology for some breeds, that is, there is genetic heterogeneity due to the selective processes used for the formation of canine breed phenotypes.

Canine DM shares similarities with the monogenic forms of diabetes known as MODY. Six genetic loci are known to be causative for this type of diabetes in humans. They are *HNF1* (Yamagata, Oda, et al. 1996), *HNF4α* (Yamagata, Furuta, et al. 1996), *glucokinase* (Froguel et al. 1992), *IPF-1* (Stoffers et al. 1997), *HNF1β* (Horikawa et al. 1997), and *NeuroD1* (Malecki et al. 1999). Given that different breeds have a definite genetic predisposition, with some breeds being at increased risk of contracting the disease while others appear to be protected (Table 3), these genes could be of interest in the dog and could identify the predisposition of certain breeds to canine DM. The possible resolution and subsequent removal of breeds suffering with monogenic forms of diabetes would result in a more homogenous population of "type 1" diabetic dogs.

In the Cavalier King Charles Spaniel, *IL-10* showed a high number of SNPs to be associated with DM. This is supportive that the gene contributes to disease susceptibility/resistance as the associations are found with more than one SNP. Given the high level of LD that exists across the canine genome, these SNPs are likely to be in strong allelic association and represent haplotypes; such heterogeneity would therefore be better assessed through haplotype analysis. Haplotype analysis in this study identified an associated haplotype in the Cavalier King Charles Spaniel with a χ^2 of 7.48 ($P = 0.005$). This haplotype did not appear to be significant in the other breeds; however, given the small numbers used, additional studies incorporating larger numbers could identify further haplotypes associated with canine DM.

Most of the cytokines associated with increased risk of developing canine DM were from the Th2 subset with *IL-4, IL-6,* and *IL-10* being predominant. This could be significant as the Th1–Th2 balance is considered to be instrumental in the development of this condition. Diabetes is believed to be initiated by Th1 cytokines and a review from 2005 (Raz et al. 2005) summarized animal model studies that had used Th2-secreted cytokines to prevent diabetes. It therefore follows that administration of Th2 cytokines (IL-10, IL-4) could be important in the prevention of T1D. To date, such treatments have not been tested in human clinical trials of T1D, but IL-10 has been used in the treatment of other autoimmune diseases including Crohn's disease (Schreiber et al. 2000) and psoriatic arthritis (McInnes et al. 2001). The identification of a number of Th2 cytokine SNPs associated with canine diabetes in this study provides support for this theory.

The insulin VNTR is reported to be associated with human T1D with the long form of the VNTR (141–209 repeats) having protective properties (Vafiadis et al. 1997) and the short form (26–63 repeats) increasing the susceptibility to T1D. The insulin VNTR is not found in the dog (Fretwell N, personal communication); however, associations were found between canine DM and insulin. Two associations were protective (INS8 and INS1, Table 4) and one inferred risk (INS8, Table 5). A protective association was also found for the canine *IGF2* gene, which is adjacent to insulin on chromosome 18. It is therefore possible that there is allelic association between these markers and that they form part of a protective haplotype.

The genetic analyses of diseases such as T1D are problematic as complex interactions exist between genetic predispositions and environmental influences. A genetic hierarchy has been identified in human populations for T1D susceptibility, of which HLA confers the greatest risk. The DLA, the canine equivalent of the MHC, has been associated with canine DM in the same diabetic dog cohort used for this candidate gene study (Kennedy et al. 2006).

Human population studies often detail hundreds or thousands of patients and controls, but associations between a marker and disease susceptibility are difficult to determine. Most canine cohorts contain only tens of patients, and large-scale recruitment of cases or controls to a study is problematic as veterinary blood samples submitted to laboratories for DNA isolation are usually residual from other diagnostic tests. This makes sample recruitment difficult, resulting in cohorts of limited size.

The alleles of a number of individual SNPs were identified in this study which showed clear association with canine diabetes. In particular, the *IL-10* gene showed a clear haplotype association to canine DM in the Cavalier King Charles Spaniel. Given that each candidate gene contributes a small amount of genetic risk to the disease, replication studies, and further haplotype analysis, would be required. Until such studies are conducted, the associations found in this study remain interesting but should be interpreted with some caution.

Acknowledgments

We are grateful to the UK Companion Animal DNA Archive who extracted DNA from many of the samples. We are also grateful to all the owners of dogs with diabetes and control dogs, who gave permission for their dogs to participate in this study.

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This paper was delivered at the 3rd International Conference on the Advances in Canine and Feline Genomics, School of Veterinary Medicine, University of California, Davis, CA, August 3–5, 2006.

Corresponding Editor: Elaine Ostrander