

## BRIEF COMMUNICATION

## Association of canine hypothyroidism with a common major histocompatibility complex DLA class II allele

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### Abstract

Dogs exhibit a range of immune-mediated conditions including a lymphocytic thyroiditis which has many similarities to Hashimoto's thyroiditis in man. We have recently reported an association in Doberman Pinschers between canine hypothyroidism and a rare DLA class II haplotype that contains the DLA-DQA1\*00101 allele. We now report a further series of 173 hypothyroid dogs in a range of breeds where a significant association with DLA-DQA1\*00101 is shown.

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There is a growing interest in investigating complex disease phenotypes in dogs, including immune-mediated (or auto-immune) diseases. This has largely been as a consequence of the completion of the sequencing of the dog genome (1–3). The dog also provides an excellent comparative model for human diseases as it spontaneously develops many similar conditions, including autoimmunity. There is a range of canine immune-mediated conditions including hypothyroidism, rheumatoid arthritis, immune-mediated haemolytic anaemia, systemic lupus erythematosus, diabetes mellitus, exocrine pancreatic insufficiency and myasthenia gravis. In a recent report by the American Kennel Club, four of the 11 most frequent canine diseases had an immunological basis,

including hypothyroidism, other immune-mediated diseases, cancer and allergic dermatitis.

Primary hypothyroidism is a common endocrinopathy in dogs (4, 5), and it is often caused by lymphocytic thyroiditis (6). Canine lymphocytic thyroiditis is considered to be an immune-mediated disease based on its clinical and histological similarities to Hashimoto's thyroiditis in man (7), and because of the prevalence of autoantibodies to thyroglobulin (8). Antibodies to circulating T3 (triiodothyronine) and/or T4 (thyroxine) also may be present (9). Definitive diagnosis of ambiguous cases is difficult even when a panel of diagnostic thyroid tests is utilized. Classical clinical signs of hypothyroidism do not usually

show up until 75% or more of the thyroid gland is destroyed. Progression of the disease eventually leads to obesity, lethargy and dermatological changes. (10). Neutered dogs, of either sex, seem to have a significantly higher risk of hypothyroidism compared with sexually intact dogs (10). The disease is typically characterized by low levels of the thyroid hormone thyroxine (T4) and its free unbound fraction (free T4), plus increased levels of thyroid-stimulating hormone (TSH). Tri-iodothyronine (T3) is rarely low and cannot be used diagnostically. Thyroid hormones may be lowered by a variety of different mechanisms, but true primary immune-mediated hypothyroidism is usually characterized by the presence of autoantibodies to thyroglobulin (11). As 50–80% of dogs with low thyroid hormone levels have autoantibodies to thyroglobulin (9, 12), any group of dogs with 'hypothyroidism' will probably be heterogeneous, adding to the difficulty in detecting a genetic association.

Immune-mediated conditions are likely to have a genetic component to their aetiology and this is supported by the fact that many such diseases display increased breed predilection or resistance. Breeds predisposed to hypothyroidism include Doberman Pinschers and golden retrievers, Borzois, giant schnauzers, Akitas, Irish setters, old English sheepdogs, Skye terriers, Shetland sheepdogs, Airedale terriers, American cocker spaniels, miniature schnauzers, pomeranians, poodles, boxers and dachshunds (4, 9, 10). An increased incidence of thyroiditis has also been reported in beagles (13) and anti-thyroglobulin antibodies in great Danes (14), English cocker spaniels (14), English pointer, English setter, German wirehaired pointer, Maltese, kuvasz and petit basset griffon Vendeen (9) although it can develop in dogs of any breed.

It is generally believed that thyroiditis is less common in mongrels. The nature, size and complexity of this genetic component are yet to be established, as are any environmental triggers. Recently, an association between canine thyroiditis and the presence of a rare DLA class II haplotype was reported for Doberman Pinschers (15). This is not surprising as genes in the major histocompatibility complex (MHC) play a central role in regulating the immune response and many human immune-mediated conditions are associated with MHC polymorphisms and haplotypes including Hashimoto's thyroiditis (16).

In dogs, the MHC is referred to as the DLA system and is known to contain class I, II and III genes. These appear to be highly polymorphic, but the full extent of this polymorphism has not been determined. We have previously investigated DLA-DRB1, DQA1 and DQB1 polymorphism in the dog and set up molecular-based methods suitable for routine DLA genotyping (17–20). To date, 67 DRB1, 21 DQA1 and 54 DQB1 alleles have been identified and named by the International DLA Nomenclature Committee (21, 22). Further DLA-DQ and DR polymorphisms have recently

been identified in the DLA workshop component of the 14th International Histocompatibility workshop (23).

We have now extended our study on canine hypothyroidism to include other dog breeds in order to examine whether the risk of developing canine immune-mediated hypothyroidism and/or thyroglobulin autoantibodies is correlated with the same specific canine MHC gene polymorphisms.

We have collected DNA samples from 173 dogs with hypothyroidism. Of these, a subset of 85 dogs has full clinical data and can be confidently diagnosed with primary hypothyroidism, based on the presence of anti-thyroglobulin antibodies of >190%, together with other clinical signs. The other 88 dogs have incomplete clinical data (notably lacking anti-thyroglobulin antibody data) and therefore may represent a more heterogeneous group.

The phenotypic and clinical data collected included sex, age and, where possible, full hypothyroid panel test results (TGAA%, TT4, TT3, FT4, FT3, autoT4, autoT3 and CTSH). The cases were compared with several different groups of control dogs: (i) all 873 typed dogs, (ii) a set of 267 matched controls (same ratio of breeds as in the 173 cases) and (iii) an exact set of 80 controls (breed-matched to 80 of the definitive cases).

Genomic DNA was extracted either using a standard phenol/chloroform method or using the Qiagen DNA Blood Mini Kit (Qiagen, Crawley, UK). All DNA was measured and normalized to a standard concentration (20 ng/μl), and stored in 96-well plates for use.

DLA-DRB1, DQA1 and DQB1 alleles were identified by sequence-based typing (SBT), by direct sequencing of purified PCR products.

Forward and reverse primers used for DLA-DRB1, DQA1 and DQB1, respectively, were – DRBF: GAT CCC CCC GTC CCC ACA G and DRBR3: CGC CCG CTG CGC TCA (15); DQAin1: TAA GGT TCT TTT CTC CCT CT and DQAin2: GGA CAG ATT CAG TGA AGA GA (24); DQB1B: CTC ACT GGC CCG GCT GTC TC and DQBR2: CAC CTC GCC GCT GCA ACG TG (24, 25).

All the primers are intronic and locus-specific. The product sizes were 303 bp for DLA-DRB1, 345 bp for DLA-DQA1 and 300 bp for DLA-DQB1.

All PCR reactions were performed with 25 ng DNA in a 25 μl reaction containing 1× PCR buffer as supplied by Qiagen (with no extra magnesium), Q solution (Qiagen), final concentrations of 0.1 μM for each primer, 200 μM each dNTP, with 2 units of Taq polymerase (Qiagen HotStarTaq). A negative control containing no DNA template was included in each run of amplifications to identify any contamination.

A standard touchdown PCR protocol was used for all amplifications, which consisted of an initial 15 min at

95°C, 14 touchdown cycles of 95°C for 30 s, followed by a 1-min annealing stage, starting at 62°C (DRB1), 54°C (DQA1) 73°C (DQB1) and reducing by 0.5°C each cycle, and 72°C for 1 min. Then 20 cycles of 95°C for 30 s, 55°C (DRB1), 47°C (DQA1), 66°C (DQB1) for 1 min, 72°C for 1 min plus a final extension at 72°C for 10 min.

All PCR samples were purified as follows: 2 units of shrimp alkaline phosphatase (USB) and 10 units of ExoI (New England Biolabs, Hitchin, UK) were added to 5 µl of PCR product, and the mixture was incubated for 1 h at 37°C, then for 15 min at 80°C. Cycle sequencing was performed using Big Dye Terminator V3 (Applied Biosystems, Warrington, UK), and samples were sequenced on an Applied Biosystems 3100 Genetic Analyzer. Sequencing data were analysed using MatchTools, and MatchTools Navigator (Applied Biosystems).

Table 1 shows the breed distribution in the 173 affected dogs, the subset of 85 putative primary hypothyroid dogs and a set of 267 breed-matched control dogs. The set of 80 controls is an exact match with 80 of the definitive cases. (This excludes five dogs for which we had no breed-matched dog). The affected dogs include only 42 different breeds, whereas our set of 873 controls contains dogs from over 70 different breeds.

There are higher numbers of affected dogs in some breeds, such as Boxer, Doberman, Rhodesian Ridgeback

and English Setter, while other breeds are not represented at all in the patients, such as Siberian Husky, Shih Tzu and Yorkshire Terrier.

We have compared the DLA allele frequencies of the patient groups with those of the control groups.

In all these comparisons, the same allele, DLA-DQA1\*00101, was shown to be significantly associated with the presence of hypothyroidism (Table 2).

The most stringent comparison of 80 dogs with definitively diagnosed immune-mediated hypothyroidism with 80 breed-matched controls gives an odds ratio of 2.57 and a *P*-value of <0.006.

There is still a highly significant association of this allele with disease in the total patient group, suggesting that the group may not be as heterogeneous as we previously thought.

Several breeds are overrepresented within this disease group, including Boxer, Doberman, Rhodesian Ridgeback and English Setter. It was difficult to find any Rhodesian Ridgebacks to use as controls, as it appears that this breed is highly susceptible to hypothyroidism.

We have looked at each of these breeds separately with regard to the presence of DLA-DQA1\*00101. It is clear from the data in Table 3 that DQA1\*00101 is raised in Dobermans, Rhodesian Ridgebacks and English setters with hypothyroidism but that it is not raised in Boxers.

**Table 1** Breed distribution of 173 hypothyroid dogs, 85 selected dogs with hypothyroidism and 267 control dogs

| Breed                                     | Hypothyroid dogs | Subset | Controls | Breed                               | Hypothyroid dogs | Subset | Controls |
|---|------------------|--------|----------|-------------------------------------|------------------|--------|----------|
| Basset Hound                              | 6                |        | 9        | Retriever (Golden) <sup>a</sup>     | 2                |        | 16       |
| Beagle <sup>a</sup>                       | 1                | 1      | 9        | Rhodesian Ridgeback                 | 26               | 20     | 15       |
| Bernese Mountain Dog                      | 1                |        | 4        | Rottweiler                          | 1                | 1      | 5        |
| Boxer <sup>a</sup>                        | 12               | 5      | 22       | Schnauzer (German)                  | 1                |        |          |
| Collie (Bearded)                          | 2                | 1      | 2        | Schnauzer (Min) <sup>a</sup>        | 1                |        | 4        |
| Collie (Border)                           | 1                |        | 2        | Setter (English Blue)               | 1                |        |          |
| Collie (Rough) X                          | 1                | 1      | 2        | Setter (English) <sup>a</sup>       | 17               | 1      | 44       |
| Dalmatian                                 | 1                |        | 3        | Setter (Gordon)                     | 2                |        |          |
| Doberman Pinscher <sup>a</sup>            | 32               | 30     | 21       | Setter (Irish) <sup>a</sup>         | 2                | 1      | 5        |
| German Shepherd dog                       | 3                |        | 14       | Sheepdog (Old English) <sup>a</sup> | 1                |        | 4        |
| Great Dane <sup>a</sup>                   | 1                |        | 4        | Sheepdog (Shetland) <sup>a</sup>    | 2                | 1      | 2        |
| Hovawart                                  | 4                |        | 3        | Spaniel (American Cocker)           | 1                |        | 2        |
| Japanese Akita <sup>a</sup>               | 1                | 1      | 2        | Spaniel (Clumber)                   | 1                |        |          |
| Labrador                                  | 4                | 3      | 14       | Spaniel (Cocker) <sup>a</sup>       | 3                | 1      | 4        |
| Labrador (Chocolate)                      | 2                |        | 2        | Spaniel (English springer)          | 2                | 1      | 4        |
| Labrador X Retriever                      | 3                | 2      | 3        | Spaniel (Springer)                  | 3                | 1      | 6        |
| Lagotto Romagnola                         | 1                | 1      |          | Spaniel (Welsh Springer)            | 1                |        | 1        |
| Leonburger                                | 1                | 1      | 1        | Terrier (Dandie Dinmont)            | 1                |        |          |
| Lhaso Apso                                | 1                | 1      | 3        | Terrier (Staffordshire Bull)        | 2                | 1      | 6        |
| Petit Basset Griffon Vendéen <sup>a</sup> | 1                | 1      |          | Terrier (West Highland White)       | 1                | 1      | 3        |
| Pointer (German Short Hair)               | 1                | 1      |          | Crossbreed                          | 6                | 3      | 12       |
| Pyrenean Mountain Dog                     | 1                |        | 4        | Unknown                             | 14               | 4      | 9        |
| Retriever (Flatcoat)                      | 1                |        | 1        | Totals                              | 173              | 85     | 267      |

<sup>a</sup>Breeds that have been reported as being predisposed to hypothyroidism. Three other predisposed breeds (Borzoi, giant schnauzer and Skye terrier) do not appear in our cohort.

**Table 2** Association of DLA-DQA1\*00101 with hypothyroidism

| Patient group     | Control group   |                           | $\chi^2$        | Odds ratio | 95% confidence interval | P-value   |           |
|-------------------|-----------------|---------------------------|-----------------|------------|-------------------------|-----------|-----------|
| All affected dogs | 105/173 (60.7%) | All controls              | 355/873 (40.7%) | 22.7       | 2.25                    | 1.59–3.19 | <0.000002 |
| All affected dogs | 105/173 (60.7%) | Breed-matched controls    | 122/267 (45.7%) | 8.87       | 1.84                    | 1.22–2.76 | <0.003    |
| Selected subset   | 57/85 (67.1%)   | Breed-matched controls    | 122/267 (45.7%) | 10.94      | 1.97                    | 1.32–2.94 | <0.001    |
| 80 cases          | 52/80 (65.0%)   | 80 breed-matched controls | 35/80 (43.75%)  | 7.48       | 2.57                    | 1.28–5.17 | <0.006    |

**Table 3** Presence of DLA-DQA1\*00101 in breeds with the highest numbers of affected dogs in this cohort

| DQA1*00101           | Doberman |          | English Setters |          | Rhodesian Ridgebacks |          | Boxers   |          |
|----------------------|----------|----------|-----------------|----------|----------------------|----------|----------|----------|
|                      | Patients | Controls | Patients        | Controls | Patients             | Controls | Patients | Controls |
| Homozygous           | 1        | 0        | 10              | 13       | 17                   | 5        | 0        | 2        |
| Heterozygous         | 14       | 4        | 6               | 13       | 8                    | 6        | 4        | 8        |
| Not present          | 17       | 26       | 2               | 7        | 1                    | 4        | 8        | 12       |
| Frequency percentage | 46.8     | 13.3     | 88.8            | 78.8     | 96.2                 | 73.3     | 33.3     | 45.4     |
| Total no. of dogs    | 32       | 30       | 18              | 33       | 26                   | 15       | 12       | 22       |

A separate analysis of a subset of these Dobermans with a different set of breed-matched controls has shown an identical trend (15).

These data suggest that MHC may influence the development of hypothyroidism in some (e.g., Doberman, Rhodesian Ridgeback and English Setter), but not all (e.g. Boxer) breeds.

Interestingly, the breeds mentioned earlier as being not represented in the patient group have low frequencies of DLA-DQA1\*00101 (Table 4). Boxers have a high frequency of a rare DLA haplotype (DLA-DRB1\*00401/DQA1\*00201/DQB1\*01501) that is not often found in other breeds. Interestingly, 11/12 (91.6%) affected Boxers have this haplotype of which six were homozygous, whereas only 14/22 (63.6%) (two homozygous) unaffected Boxers had it. While these data are not significant, they are suggestive of a different MHC association.

Although our data set includes dogs from 42 different breeds, many breeds are present in low numbers while only four breeds are present in larger numbers. To some extent, this is influenced by how the samples were collected (some breed clubs), and it remains unclear whether these data represent a true cross-section of dogs affected by this disease. Thirteen of the 16 reported predisposed breeds occur

in our data set, and only three of those (Boxer, Doberman pinscher and English setter) appear in high numbers. We continue to collect samples from many different sources, so as to increase numbers in each breed.

Our data suggest that some dog breeds may have MHC associations with thyroiditis that are different from MHC associations in other breeds and that some may even lack a strong MHC association. This is in keeping with previous findings in human immune-mediated thyroid disease where a number of different associations both within (26) and between ethnic groups (27, 28) have been found. The explanation for observing multiple MHC associations is unclear, but one possibility may be the existence of other MHC-linked disease genes in linkage disequilibrium with the class II alleles and haplotypes associated with thyroiditis. One such contender is the pro-inflammatory cytokine TNF $\alpha$ , and support for this possibility has recently been provided (29).

Previous genetic linkage and association studies of human immune-mediated thyroid conditions have provided evidence for the contribution of non-MHC susceptibility gene regions and loci (30). These include the CTLA-4 gene (31), CD40, thyroglobulin and the immune-mediated regulator gene on chromosome 21 (30). We now intend to examine whether such potential susceptibility genes are candidate genes in the dog. The baseline MHC association data we have identified in dog breeds should assist in this process.

**Table 4** Frequency of DLA-DQA1\*00101 in possible resistant breeds

| Breed             | Number of dogs | Frequency (%) of DLA-DQA1*00101 |
|-------------------|----------------|---------------------------------|
| Siberian Husky    | 16             | 6.3                             |
| Shih Tzu          | 27             | 3.7                             |
| Yorkshire Terrier | 31             | 9.6                             |

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