Analysis of the Inheritance of White Spotting and the Evaluation of KIT and EDNRB as Spotting Loci in Dutch Boxer Dogs


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Abstract

The genetic basis of the white spotting pattern in Dutch boxer dogs is not known. We studied whether the segregation of white spotting in boxers follows a Mendelian inheritance pattern. Blood samples were collected, along with digital photographs in standard directions of (grand)parents (n = 16) and offspring (n = 52) from eight litters of Dutch boxers. In order to select heterozygous parents, we selected nonuniform litters, in which at least one puppy was extreme white. On the basis of criteria for the location, the extent of white spotting, and the mean percentage of pigmented area of the foot soles, we classified 10 dogs as solid colored, 27 as flashy, and 15 as extreme white. This was not a significant deviation from the expected 1:2:1 ratio. Because the flashy phenotype seems to be an intermediate between the two homozygotes, white spotting in the Dutch boxer can be considered to be due to a single gene effect, with incomplete dominance. We have evaluated candidate genes c-KIT (KIT) and EDNRB for segregation with white spotting phenotype in these litters. Using polymorphic markers, very near the KIT and EDNRB genes, we found that segregation of the white spotting pattern did not coincide with segregation of these polymorphic markers. Thus neither KIT nor EDNRB are likely to be responsible for white spotting in the Dutch population of boxers.

In domestic animals the major locus of white spotting is known as the spotting locus (S). This locus comprises, in decreasing order of dominance, the S, s', s^p, and s^w alleles. S stands for solid coat color, s' for Irish spotting, s^p for piebald spotting, and s^w for extreme white (Little 1957). The S series alleles appear to be incompletely dominant. The genotypes Ss^w and s's can produce the (pseudo-)Irish spotting phenotype, characterized by more or less extended white markings at the muzzle, forehead, neck, chest, belly, one or more feet, and the tail tip (Little 1957). White markings are caused by differences in the maturation and survival of melanocytes (Bennett and Lamoreux 2003; Rawls et al. 2001) that are derived from the neural crest cells. The immature melanocytes must migrate from the dorsal midline over the surface of the animal to the periphery, which is why the areas furthest from the dorsal midline (feet, chest, muzzle) are most likely to have white spots. Although it has been postulated that all white spotting in dogs is due to the same major locus, allelism tests between the spotting phenotypes in all the different dog breeds have not been performed (Metallinos and Rine 2000). According to the breed standard for boxer dogs, only less than one-third of the coat is allowed to be white (American Kennel Club 1997). It is assumed that the s^p allele does not exist in the boxer breed (Little 1957; Robinson 1990). In the British boxer dog population, the s' allele seems to have disappeared as well (Cattanach 2001).

White spotting loci have been characterized at the molecular level in several species. More than 20 different mouse mutants exist that display hypopigmentation defects (Silvers 1979). The proteins encoded by the genes altered in these mutants range from transcription factors to ligand/receptor signaling molecules (Rhim et al. 2000). The phenotypic effect of the mutations depends on the kind of lesion and whether the mutation is heterozygous or homozygous. Next to a characteristic white spotting phenotype, the mutant homozygotes occasionally show
Breeders were asked emphatically if puppies were stillborn, extreme white, while none of the parents was extreme white. Parents, we selected litters in which at least one puppy was to participate in our study. In order to select heterozygous markers for KIT and EDNRB was performed with litters of Dutch boxers in which at least one dog with an extreme white phenotype was present.

Materials and Methods

Animals

Several breeders from the Dutch Boxer Club were contacted to participate in our study. In order to select heterozygous parents, we selected litters in which at least one puppy was extreme white, while none of the parents was extreme white. Breeders were asked emphatically if puppies were stillborn, euthanized, or had died before our visit. In boxers, the relationship of coat color and risk of death has not been described. Therefore we assumed a similar risk of death in all three genotypes, and we excluded phenotypes of stillborn or early deceased puppies (no puppies were euthanized). Data for 52 puppies from eight litters were collected, together with the data for 13 different parents of those puppies. One sire was the father of three different litters.

DNA Isolation

Blood samples from the extreme white boxers, their available littermates, and their parents were collected. We could not collect blood samples from the male parent of litters 5 and 6. Genomic DNA was isolated by the salt extraction method of Miller et al. (1988).

Photographs

Photographs were taken with a digital camera according to a standard procedure. We photographed both lateral sides, while the standing dog was looking forward, with all four feet visible. Next we photographed the front of the dog while standing or sitting, and the back. Photographs of the belly and chest were taken while the dog was standing on its hind feet and two people held the front feet displaying the armpits. Finally, photographs were taken of the footpads to determine the extent of pigmentation.

Classification of the Spotting Genotype

The parents and offspring were classified in one of three genotype groups: SS, Ssw, or Sswo, based on their white spotting pattern. The Ssw group consisted of the easily recognized extreme white boxers, which are either totally white or have one or a few pigmented specks. To study whether the white spotting pattern of solid and flashy boxers can be distinguished, a classification system was developed. We categorized these dogs by scoring the presence of six characteristic features of white spotting in flashy boxer dogs, as listed in Table 1. If one of the features was present, the dog scored one point. Hence the total score in our classification system ranged from zero to six points. The total pigmented area of the front and hind footpads (in percentages) was estimated after each dog was classified in our classification system.

Table 1. Criteria to classify Dutch boxers with minor and medium white spotting

<table>
<thead>
<tr>
<th>Phenotypic features</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>White spotting proximal of the metacarpophalangeal/metatarsophalangeal joint.</td>
</tr>
<tr>
<td>B</td>
<td>White spotting over 50% of the following surface: the ventral side of the lower jaw, the ventral side of the neck, and the chest up to and including the area between the front legs, seen from the front.</td>
</tr>
<tr>
<td>C</td>
<td>White spotting on the muzzle extending underneath the nasal planum.</td>
</tr>
<tr>
<td>D</td>
<td>White stripe at the forehead coat extending above the eyes.</td>
</tr>
<tr>
<td>E</td>
<td>White spotting on the lateral and/or dorsal side of the neck.</td>
</tr>
<tr>
<td>F</td>
<td>White spotting caudal of the front legs on the chest and/or belly.</td>
</tr>
</tbody>
</table>

Phenotypic features each score one point.

Although there are many different dog breeds that segregate white spotting patterns, no genes have been identified that are linked to these phenotypes (Metallinos et al. 1998, Metallinos and Rine 2000). EDNRB and KIT were excluded as the basis for piebald spotting in border collies (Metallinos and Rine 2000). EDNRB and KIT were excluded as the basis for piebald spotting in border collies (Metallinos and Rine 2000). EDNRB and KIT were excluded as the basis for piebald spotting in border collies (Metallinos and Rine 2000). EDNRB and KIT were excluded as the basis for piebald spotting in border collies (Metallinos and Rine 2000). EDNRB and KIT were excluded as the basis for piebald spotting in border collies (Metallinos and Rine 2000). EDNRB and KIT were excluded as the basis for piebald spotting in border collies (Metallinos and Rine 2000). EDNRB and KIT were excluded as the basis for piebald spotting in border collies (Metallinos and Rine 2000).

A pleiotropic effect (Oetting and Bennett 2004). Well-characterized examples of white spotting loci in the mouse are dominant white spotting, due to mutations in the gene encoding the type III receptor tyrosine kinase (KIT), and piebald, due to mutations in the G-protein coupled endothelin receptor B (EDNRB). KIT and EDNRB seemed good candidate genes for the spotting locus in Dutch boxer dogs because mutant alleles show similar phenotypes in other species. Mutations of KIT have been described in the pig (Guiffrà et al. 1999, Hirooka et al. 2002, Johansson Moller et al. 1996, Markland et al. 1998, Pieberg et al. 2002) and cattle (Grosz and MacNeil 1999; Olsen et al. 2000; Reinsch et al. 1999). Lethal white foal syndrome in horses is caused by a missense mutation in the EDNRB gene (Metallinos et al. 1998).

We tested the hypothesis that the white spotting pattern in Dutch boxers is due to a single gene effect, clearly expressing three different phenotypes: solid color, flashy, and extreme white. A genetic linkage study with polymorphic markers for KIT and EDNRB was performed with litter sizes of Dutch boxers in which at least one dog with an extreme white phenotype was present.

Statistics

The chi-square test was used to determine if the observed ratio between groups differed significantly from the expected ratio (critical value 5.99; \( F_{2,0.05}[2] = 5.99 \)). The percentage of pigmented area (mean ± SD) of the front feet, the hind feet, and all four feet was calculated for each genotypic group and for each individual. The mean percentages of pigmented area for the four feet in the three genotype groups were compared using the analysis of variance (ANOVA) table in...
SPSS 10.0 for Windows (the F test). The Student t test was carried out to compare the mean of the percentages of pigmented area for the four feet of each genotype group to another.

**Microsatellite Analysis**

The segregation of KIT was evaluated with the polymorphic GAAA repeat developed by Metallinos and Rine (2000). The polymerase chain reaction (PCR) oligonucleotide sequences were 5'-GCATGGACCTGCTTCTCG-3' (KIT forward) and 5'-AGAGCATCTTTGGTGTTGCGC-3' (KIT reverse). The forward primer was labeled with the HEX fluorophore. The segregation of EDNRB was studied with the polymorphic GA repeat described by Zemke and Yuzbasiyan-Gurkan (1999) and with oligonucleotides based on the microsatellite marker REN69E24. The latter marker is located close to EDNRB according to the recent map of the dog genome (Guyon et al. 2003). The PCR primer sequences were, respectively, 5'-GAGAATTGGCGATGCAGGA-3' (EDNRB forward), 5'-AGAGGCTTCTCAGCTTCTCG-3' (EDNRB reverse), 5'-GGATGCGAGTAAAGAAGATGC-3' (REN69E24 forward) and 5'-AGTCACTGTCAGCTGGGACCA-3' (REN69E24 reverse). The forward primers of both the EDNRB markers were labeled with the 6-FAM fluorophore.

For each PCR reaction, 25 ng of genomic DNA was used as template in a 15 μl reaction consisting of 1×Gibco-BRL buffer (Life Technologies), 1.5 mM MgCl₂, 0.5 μM of each primer, and 0.6 U platinum Taq polymerase (Life Technologies). The PCR program consisted of an initial denaturation step of 10 min at 94°C, amplification was for 35 cycles (30 s at 94°C, 30 s at 55°C [KIT 65°C], and 30 s 72°C) followed by a 10 min extension at 72°C. PCR products were diluted 50 times with water and 1 μl of the diluted with 10 μl formamide and 0.15 μl of the size standard GS-400-HDROX (Applied Biosystems) and analyzed on the ABI 3100 Genetic Analyzer (Applied Biosystems) with filter set D. The alleles were scored with GENESCAN software. The linkage was analyzed using MLINK of the LINKAGE software package. The phenotype was coded as a locus with numbered alleles, assuming flashy dogs to be heterozygous and solid color and extreme white dogs to be homozygous with different alleles.

**Results**

**Classification of the Spotting Genotype**

We collected data for 52 puppies and their parents from 8 nonuniform litters in which 1 or more white puppies were born. Fifteen pups were extreme white (s⁺w⁺) and 37 showed minor or medium white spotting. When these 37 puppies were classified according to the extent and location of white coat color (Table 1), a bimodal distribution was observed. The minor white spotted group (solid, SS, n = 10) scored zero (three cases), one point (six cases), or two points (one case). When present, the white marking in this group was seen only on the forehead (feature D) and chest/belly (feature F). The medium white spotted group (flassy, Ss⁺, n = 27) scored six points (18 cases), five points (8 cases), or three points (1 case). Feature B, more than 50% white from lower jaw to front chest, was exclusively seen in this medium white spotted group. Fifteen of the 16 parents scored six points. One parent scored four points, but still displayed feature B, so all parents could indeed be classified in the Ssw group.

**Segregation Analysis of the White Spotting Phenotype**

The observed ratio of solid color, flashy, and extreme white among offspring was 10:27:15. Assuming that flashy Dutch boxers have the Ssw genotype, we expected a genotype distribution of 1:2:1 (SS: Ssw: swsw) in offspring of the selected heterozygous parents. The observed ratio was not significantly different from the expected ratio of 13:26:13 (χ² = 1038). Therefore our hypothesis, that the observed ratio of white spotting in boxers can be explained by one gene with incomplete dominance, was not rejected (P < .05).

The percentage of pigmented area of both the front and hind footpads was estimated in those dogs whose footpads were photographed (n = 66). The mean (± SD) percentage pigmented area of the front and hind footpads in the SS group (n = 10) was 86.9 (± 10.6), in the Ssw group (n = 41) it was 24.9 (± 20.0), and in the swsw group (n = 15) it was 0.5 (± 0.9). The F test (ANOVA) was carried out and a significant difference among groups was found: F = 86.07 > F₀.₀.₅ [2.63] = 3.12. The Student t test revealed a significant difference between the SS and Ssw group (t = 9.44 > t₀.₀.₅ [50] = 2.009), between the Ssw group and swsw group (t = 4.71 > t₀.₀.₅ [55] = 2.009), and between the SS and swsw group (t = 31.90 > t₀.₀.₅ [24] = 2.064).

**Microsatellite Analysis**

Initially we determined whether the available sires and dams of the seven litters were informative for linkage analysis of the microsatellite markers. Two allele lengths of 224 bp and 228 bp were observed for the KIT marker and heterozygous parents. The observed ratio was not significantly different from the expected ratio of 13:26:13 (χ² = 1038). Therefore our hypothesis, that the observed ratio of white spotting in boxers can be explained by one gene with incomplete dominance, was not rejected (P < .05).

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of 0.6 at recombination fraction 0.01. The REN69E24 marker did not segregate with the spotting locus. The two-point LOD score was 1.6 at recombination fraction 0.01.

The physical distance between the marker and EDNRB has been calculated at 0.5 Mb (Guyon et al. 2003) and the genetic distance was therefore estimated to be less than 1 cM. Assuming this close spacing, the combined three-point LOD score between the EDNRB marker, REN69E24, and the S locus was 1.0.

**Discussion**

Data for 52 puppies from eight litters were collected. Because each litter contained at least one puppy with an extreme white phenotype, all parents were assumed to be heterozygous. The obtained ratio of phenotypes of puppies in eight boxer litters was not significantly different from the expected ratio 1:2:1, which is in line with a one-gene effect with incomplete dominance. In addition, the percentage of pigmented area of the foot soles in boxers with an assumed SS, Ssw, or ssw genotype differed significantly. The white marking, when present, in the solid SS group, was seen only on the forehead and chest/belly. These specific markings therefore may be explained by the influence of another gene, as is presumed in other species. In the rat, a mutant allele of a new white spotting locus has been described as head spot (Robinson 1998). In horses, white markings on the forehead are assumed to be influenced by alleles at different loci acting in a cumulative manner (Woolf 1992).

Linkage analysis showed that KIT segregated independently from the spotting locus. The LOD score of −3.4,
below the threshold value of −2 for exclusion, excludes KIT as a candidate gene. Although others examined the KIT locus for involvement in white spotting in dogs (Metallinos et al. 2000), no other study focused on a dog breed for which white spotting is described as part of the breed standard. Our findings complement the results of Metallinos and Rine (2000), who excluded KIT as a cause of white spotting pattern (spsp) in border collies. However, the combined three-point LOD score between the EDNRB marker, REN69E24 marker, and the S locus was −1.0, indicating that it is highly unlikely that the gene is involved.

Rhim et al. (2000) describe that KIT and EDNRB can also act synergistically. They found two inbred mouse strains, heterozygous for Ednrbs and KitWj−2, which were phenotypically 0% and 8.5% white, respectively. Double heterozygous animals were 58.1% white and Ednrbs/Ednrbs/KitWj−2/+ animals were almost completely white. This white coat color distribution is similar to the distribution in our boxer litters. However, the EDNRB segregation data presented here do not support such a synergistic action of EDNRB and KIT.

Perspectives

There are a still a number of spotting loci to evaluate (Bennett and Lamoreux 2003). The spotting genes are most likely involved in a network regulating the lineage determination, migration, and proliferation of the melanoblast/melanocyte. In addition to the ligand/receptor interactions, other interactions between these loci have been discovered; for example, SOX10 and PAX3 influence the activity of MITF, which in turn can act as a promoter of KIT transcription (Potterf et al. 2000; Wu et al. 2000). In humans, mutations in SOX10, PAX3, and MITF are responsible for different types of the Waardenburg-Shah syndrome that is associated with sensorineural deafness and white forelock (Bondurand et al. 2000; Potterf et al. 2000; Spritz and Beighton 1998). Like in other dog breeds, pigment-associated congenital hereditary sensorineural deafness has been reported in boxers (Strain 1999). Of particular interest is the steel locus, encoding MGF, the EDNRB ligand. In inbred mice it has been shown that this gene or a gene in the direct vicinity is a major recessive modifier of white spotting in Ednrbs/Ednrbs mice (Rhim et al. 2000). Remarkably, one allele of this locus is semidominant and is associated with the penetrance and expressivity of a white forelock phenotype, similar to that seen in our SS group of boxers. With the genomic map of the dog soon available, it should be possible to elucidate the molecular basis of white spotting in the boxer and in many other dog breeds.

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