A New Mutation in *MC1R* Explains a Coat Color Phenotype in 2 "Old" Breeds: Saluki and Afghan Hound

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Melanocortin 1 Receptor (MC1R) has been studied in a wide variety of domestic animals (Klungland et al. 1995; Marklund et al. 1996; Våge et al. 1997; Kijas et al. 1998; Newton et al. 2000; Våge et al. 2003), and also several wild animals (Robbins et al. 1993; Ritland et al. 2001; Eizirik et al. 2003; Nachman et al. 2003; McRobie et al. 2009) in relation to coat color variation. A variety of phenotypic changes have been reported including coat colors from pure black to pure red, as well as some phenotypes with hairs with red and black bands. One phenotype, called grizzle in Salukis and domino in Afghan Hounds, appears to be unique to these 2 old dog breeds. This pattern is characterized by a pale face with a widow's peak above the eyes. The body hairs on the dorsal surface of Salukis and Afghan Hounds have both phaeomelanin and eumelanin portions, even though they had an a^t/a^t genotype at *ASIP*. In addition, all had at least one copy of a newly identified mutation in *MC1R*, g.233G>T, resulting in p.Gly78Val. This new allele, that we suggest be designated as E^G, is dominant to the *E* and *e* (p.Arg306ter) alleles at *MC1R* but recessive to the E^M (p.Met264Val) allele. The K^B allele (p.Gly23del) at *DEFB103* and the a^y allele (p.Ala82Ser and p.Arg83His) of *ASIP* are epistatic to grizzle and domino.

Key words: *Afghan hound*, ASIP, *coat color, domino, grizzle,* MC1R, *pigmentation, Saluki*

The Saluki and Afghan Hound are both considered "old" breeds based on several genetic studies (Parker et al. 2004; Boyko et al. 2009) and are also closely related. Both Salukis and Afghan Hounds occur in a wide variety of coat colors (Supplementary Table 1). Over the course of time, different colors have been selected more in one breed than the other and so the allele frequencies are not the same in both breeds.

Both breeds share a coat color pattern that has not previously been studied using molecular genetics. This pattern is now known as domino in Afghans, after a famous show dog of that name. The pattern is called grizzle in Salukis, presumably because the dorsal surface looks like it contains a mix of eumelanin and phaeomelanin.

In the course of a coat color study of Salukis, we became interested in the genes involved in these patterns, as well as their inheritance, which has been poorly understood.

Materials and Methods

Dogs and Families

DNA was obtained for this study from a number of Salukis and Afghan Hounds of various coat colors, including grizzle or domino pattern, using cheek brushes (Epicentre, Madison, WI). The Salukis were collected widely, from several countries. The Afghan Hounds were collected only in North America and a proportion of them represent a large multigeneration family that helped to verify the inheritance pattern. In addition, DNA from our bank of dogs studied previously in other coat color studies was utilized. Photographs of the dogs studied were used to verify the coat color and pattern. Hair samples were also examined in some cases. The dogs are owned by private individuals and signed consent from the owners was obtained for use of their DNA in coat color studies, in keeping with Canadian Animal Care guidelines.

Hair Analysis

Hair samples were pulled from the hip and shoulder area of some dogs that were grizzle or domino pattern. Individual hairs were examined under a dissecting microscope to confirm the root end and to check the distribution of phaeomelanin and eumelanin color.

DNA Sequencing

Because *Melanocortin 1 Receptor (MC1R)* is a single exon gene, we were able to obtain the entire coding sequence from genomic DNA. Primers D and E (Newton et al. 2000) were used for amplification of a 1262-bp fragment. Polymerase chain reaction products were isolated using the QIAquick method (Qiagen, Mississauga, ON) and were sequenced at

the National Research Council of Canada Plant Biotechnology Institute using an ABI Prism 373 Sequencer (Perkin Elmer Corporation) and the Big Dye Terminator kit (Perkin Elmer Corporation). Sequences were aligned using the Sequencher 4.8 computer software (Gene Codes Corporation, Ann Arbor, MI).

Genotyping

All individuals were genotyped for the presence of the a^y allele of *ASIP* using our previously published method (Berryere et al. 2005). A proportion of individuals were also genotyped for the *a,* or recessive black, allele of *ASIP* (Kerns et al. 2004), although this allele is atypical outside of herding breeds.

Previously reported protocols were used to detect the *e* (Schmutz et al. 2002) and E^M alleles (Schmutz et al. 2003) of *MC1R*. A protocol was designed to detect the new *MC1R* mutation.

Results

Hair Analysis

The hairs from grizzle Salukis were typically a pale phaeomelanin shade at the base or root end and a dark eumelanin color at the tip. The proportions of phaeomelanin and eumelanin varied in different areas of the body but no hairs had alternating bands of phaeomelanin and eumelanin. There were some guard hairs that were totally a eumelanin color. The shorter or undercoat hairs were a pale cream color.

The hairs from domino Afghans were much longer and softer and had alternating bands of phaeomelanin and eumelanin of varying size on the same individual and among individuals. They also had some hairs with a phaeomelanin based and eumelanin at the terminal end.

MC1R Sequence Analysis

A previously unreported mutation was discovered in the *MC1R* sequence of several Salukis. The mutation is a g.233G>T substitution, resulting in an amino acid change from a glycine to valine at residue 78 (p.Gly78Val) (GenBank GU220379) which we have named E^G. Dogs that were heterozygous and homozygous for valine were identified. This mutation was observed only in Salukis (GenBank GU220378) and Afghan Hounds (GenBank GU220379) (Supplementary Table 2) and not in any dogs of other breeds that were examined including Malamute, Shetland Sheepdog, and Siberian Husky.

Other mutations were also detected in the *MC1R* sequence. Some, such as p.Arg306ter, known as the *e* allele (Newton et al. 2000) and p.Met264Val, known as the E^M allele (Schmutz et al. 2003) were observed (Supplementary Table 2). Other previously reported substitutions, not associated with a particular coat color phenotype, such as g.627C>T (Newton et al. 2000) were also observed. In addition some SNPs, not previously reported or available

in GenBank sequence, were observed. These included g.−45A>G in the 5′ region (GenBank GU233654) and g.*184G>C in the 3′ region (GenBank GU233655), as well as a g.476C>A which resulted in a proline to glutamine change in residue 159 (p.Pro159Gln) (GenBank GU233656). All 3 of these variants were detected in Malamute and Siberian Husky, as well as in Saluki and Afghan Hound (Supplementary Table 3).

Haplotype analysis showed that the −45A allele was only present in dogs that also had a copy of 159Q. However, not all dogs homozygous for 159Q were homozygous for −45A. These 2 alleles may be in linkage disequilibrium (LD) but they are not in complete LD. None of the previously reported mutations or the newly discovered variants were in complete LD with the 78V allele (Supplementary Table 3).

Phenotypic Association

The 2 coordinators of a recent study we conducted on coat color in Saluki, Margot Keast and Casey Gonda, developed the following definition: "Grizzle—A pattern that is present at birth and remains throughout life, which comprises a dark overlay covering the top and sides of the body and outside of the limbs, from the top of the head to the tip of the tail. The darker color on the head gives the impression of a 'widow's peak' between the eyes where it contrasts with the lighter color of the face. The underside of the dog and the inner sides of the legs are the lighter base color. The overlay color may be black, gray/silver, red, or chocolate and can vary in intensity with the seasons and age. The base color may be various shades of red, fawn, golden, cream, or silver."

The common characteristic that is most easily seen in grizzle Salukis (Figure 1A,B) and domino Afghan Hounds (Supplementary Figure 1A,B) is the pale face with a pronounced widow's peak pattern above the eyes. We suggest that the new 78V allele be called E^G, consistent with other allele terminology used by dog breeders and owners since the classic work of Little (1957) who referred to this phenotype as grizzle in both Afghan Hounds and Salukis.

Not all the dogs with a 78V, or the E^G allele, had a grizzle or domino phenotype. Only dogs that also had an a^t/a^t genotype at *ASIP* had this phenotype. A single E^G allele in the presence of an *E* or *e* allele, in combination with an a^t/a^t genotype was sufficient to result in a grizzle or domino phenotype (Supplementary Table 1), except in black dogs with a K^B allele. This suggests that the E^G allele is dominant to both the *E* and *e* allele. Both Saluki and Afghan Hounds commonly have an a^y allele at *ASIP*, which is dominant to the a^t allele, and this a^y allele is epistatic to grizzle and domino. Neither the a^w nor the *a* allele are present in either breed (Dreger DL, Schmutz SM, Unpublished data).

Several Afghan Hounds we studied had an E^M allele and the E^G allele (Supplementary Table 2). These dogs had a melanistic mask over most of their muzzle. If they were also a^t/a^t, they had tan or pale eyebrow spots. Their body

Figure 1. Photos of Salukis representing the grizzle phenotype (**A** and **B**) and the eumelanin-and-tan in both the brown-and-tan (**C**) and black-and-tan (**D**) variations.

color was also consistent with a black-and-tan pattern (Supplementary Figure 1C,D). Therefore, this suggests that the E^M allele is dominant to the E^G allele. A single Saluki had both an E^M allele and E^G allele and it also had a black-and-tan pattern on its body.

Dogs that have a K^B allele at *DEFB103* (Candille et al. 2007) are black, brown, or gray. This allele is epistatic to the alleles of *ASIP* and such black dogs did not show a grizzle pattern (Supplementary Table 2). The K^B allele is relatively common in Afghan Hounds and relatively rare in Salukis. However, a few black dogs in each breed were included in this study.

Family Studies

A large multigeneration family of Afghan Hounds that included dogs that were domino, black-and-tan, fawn/sable, and cream was also studied. As predicted by the results in the individual Salukis, the inheritance of domino requires that both an a^t/a^t genotype at *ASIP* and the presence of at least one copy of the 78V mutation, or E^G allele, and no E^M or K^B allele are present (Figure 2). Therefore, the inheritance pattern does not follow a simple single gene mode of inheritance.

Discussion

Little (1957) mentioned that the grizzle pattern occurred in Saluki. He suggested that it was likely a modification of sable, now known to be caused by an a^y allele. This study provides evidence that it would more appropriately be called a modification of black-and-tan. He stated that he had not studied Afghan Hounds sufficiently to postulate the alleles involved in their various colors.

Domino has been considered a rare pattern in Afghan Hounds. The pattern began to be referred to as "domino" because of a dog of this pattern named Tanjores Domino, born in Sweden on 10 July 1954 and imported to the United States, who did well in show circles. Prior to his popularity, it was called reverse mask by fanciers of Afghan Hounds. The rarity of the domino pattern can now be better understood because this pattern requires the presence of a specific combination of alleles at more than one locus and not simply a single allele at one locus. Furthermore, the E^M allele was very common in Afghan Hounds we studied (Supplementary Table 2).

Malamutes and Siberian Huskies have a similar facial pattern and are also considered to be old breeds relatively closely related to the Afghan Hound and Saluki (Parker et al.
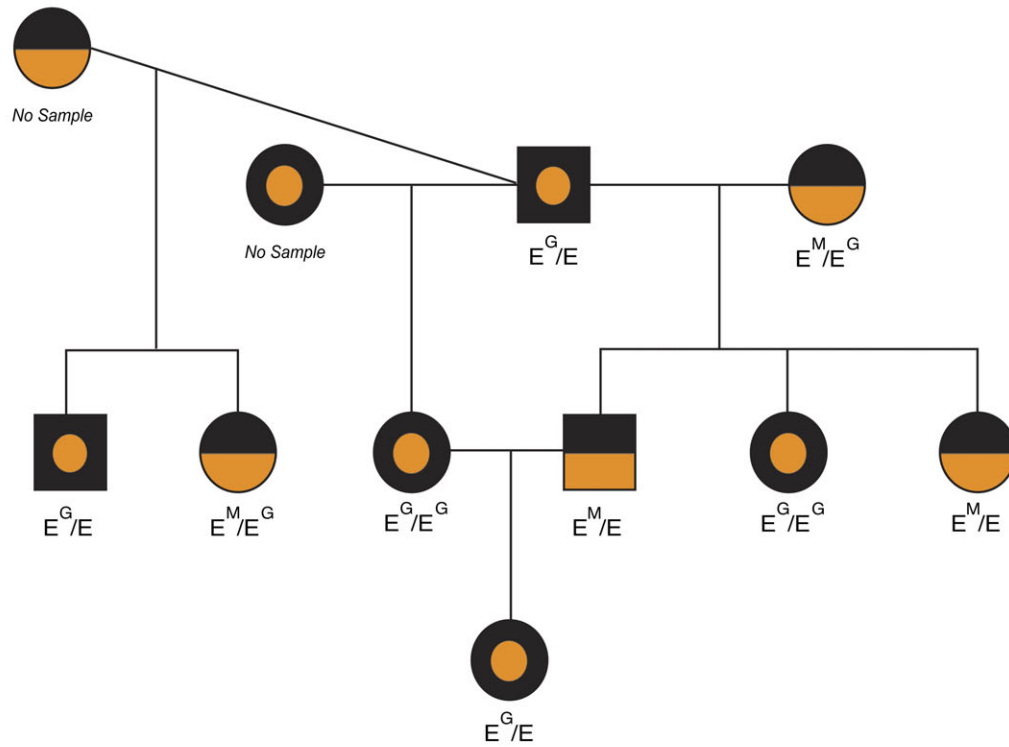
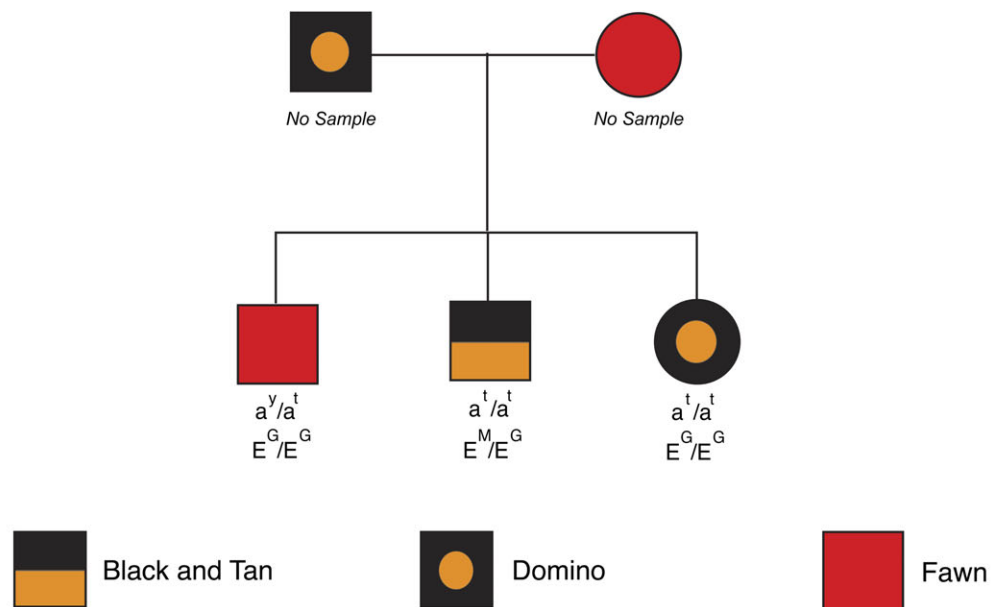**Figure 2.** (**A**) A pedigree of an Afghan Hound family, all members of which are d^t/d^t at *ASIP* and k^y/k^y at *DEFB103* illustrating that the E^M allele is dominant to the E^G allele. Dogs with an E^G/E^G or E^G/E genotype are domino pattern. (**B**) A pedigree of an Afghan Hound family that illustrates that the a^y allele at *ASIP* causes dogs to be cream to fawn, rather than domino, even with an E^G/E^G genotype.

2004), but neither breed had the 78V allele. Some Shetland Sheepdogs and Collies have a facial pattern reminiscent of the widow's peak appearance in Salukis and Afghan Hounds but such dogs did not have the 78V allele either.

There are dogs that are close relatives of the Saluki, known as the Sloughi and the Tazi. Dogs of these breeds may have this allele, but we did not have dogs from these breeds in our study.

The 78th amino acid is highly conserved across species, as a cysteine. However, in most dogs, this amino acid is a glycine and in the grizzle and domino dogs, it is a valine. Based on the postulated structure of MC1R, this amino acid is the last amino acid on the intracellular surface near the start of the second transmembrane domain. The amino acids adjacent to the membrane appear to be important based on mutations reported in other species which affect phenotype. Based on this same 7 transmembrane structure (Garcia-Borrón et al. 2005), the changed from glycine to valine would dramatically alter the folding of *MC1R mRNA*, based on the 2D folding model, Quikfold (http://dinamelt.bioinfo .rpi.edu/quikfold.php).

Various point mutations that cause amino acid changes, deletions that are in or out of frame, and premature stop codons in *MC1R* have been reported to cause red or black coat color in several animals such as cattle (Klungland et al. 1995), horse (Marklund et al. 1996), pig (Kijas et al. 1998), dog (Newton et al. 2000), sheep (Våge et al. 2003), jaguar, and jagarundi (Eizirik et al. 2003). The E^M allele in dogs, which is caused by a g.799A>G, resulting in a valine instead of a methonine at amino acid 264 is more unusual. Eumelanin pigmented hairs are prominent on the face and sometimes also on the tail tip.

In some mammals, including most rodents, the hairs are typically composed of phaeomelanin and eumelanin bands. Some *MC1R* mutations alter the width or placement of these bands. The G78V mutation, or E^G allele, of the present study, fits better into this group of mutations. However, banded hairs occur instead of solid eumelanin pigmented hairs on the dorsal surface in grizzle Salukis and domino Afghan Hounds. McRobie et al. (2009) have reported differences in the size of phaeomelanin and eumelanin bands in the gray squirrel caused by a 24-bp deletion that includes amino acids 87–94. This mutation acts in a codominant fashion with squirrels homozygous for the deletion appearing very dark to black and squirrels heterozygous for the deletion appearing intermediate in shade. Nachman et al. (2003) reported that pale pocket mice had a subterminal band of phaeomelanin, whereas dark mice did not. This phenotypic difference is similar to the grizzle pattern in Salukis.

However, the feature that is more difficult to associate with this *MC1R* mutation is the characteristic pale face with widow's peak. Given that the E^G allele only causes the grizzle or domino pattern with the widow's peak face in dogs that would otherwise be eumelanin-and-tan with an a^t/a^t genotype, we suggest that this allele minimizes the extent of the eumelanin dorsal area in such dogs. The underside color is extended up over the chest of the dog onto the face.

The underside color also covers the full ventral surface, rather than the 2 chest spots and lower legs typical of most eumelanin-and-tan dogs. This mutation also appears to affect the binding of MSH and/or ASIP to MC1R because the dorsal surface of dogs with an a^t/a^t genotype at *ASIP* would normally consist of solid eumelanin pigmented hairs and in the presence of the E^G allele these hairs also have phaeomelanin pigmented portions.

Supplementary Material

Supplementary material can be found at http://www.jhered .oxfordjournals.org/.

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