A non-coding regulatory variant in the 5'-region of the *MITF* gene is associated with white spotted coat in Brown Swiss cattle

Sonja Hofstetter[1], Franz Seefried[2], Irene Häfliger[1], Vidhya Jagannathan[1], Tosso Leeb[1], Cord Drögemüller[1,*]

[1]Institute of Genetics, Vetsuisse Faculty, University of Bern, 3001 Bern, Switzerland

[2]Qualitas AG, 6300 Zug, Switzerland

*corresponding Author:

Cord Drögemüller

Institute of Genetics

Vetsuisse Faculty

University of Bern

Bremgartenstrasse 109a

3001 Bern

Switzerland

Phone: +41 31 631 25 29

Fax: +41 31 631 26 40

E-mail: cord.droegemueller@vetsuisse.unibe.ch

Summary

Recently, the Swiss breeding association reported an increasing number of white spotted cattle in the Brown Swiss breed which is normally solid brown coloured. A total of 60 Brown Swiss cattle with variably sized white abdominal spots, facial markings and depigmented claws were collected for this study. A genome-wide association study using 40k SNP genotypes of 20 cases and 1619 controls enabled us to identify an associated genome region on chromosome 22 containing the *MITF* gene encoding the melanogenesis associated transcription factor. Variants at the *MITF* locus have been reported before to be associated with white or white-spotted phenotypes in other species such as horses, dogs and mice. Whole genome sequencing of a single white spotted cow and subsequent genotyping of 172 Brown Swiss cattle revealed two significantly associated completely linked single nucleotide variants (rs722765315 and rs719139527). Both variants are located in the 5'-regulatory region of the bovine *MITF* gene and comparative sequence analysis showed that the variant rs722765315, located 139 kb upstream of the transcription start site of the bovine melanocyte specific *MITF* transcript, is situated in a multi-species conserved sequence element which is supposed to be regulatory important. Therefore we hypothesize that rs722765315 represents the most likely causative variant for the white spotting phenotype observed in Brown Swiss cattle. Presence of the mutant allele in heterozygous or homozygous state supports a dominant mode of inheritance with incomplete penetrance and results in a variable extent of coat colour depigmentation.

Keywords

Coat colour, depigmentation, *Bos taurus*, *MITF*, melanocyte

Introduction

White spotting coat colour phenotypes are well known in many species. At present, there are already eight genes known to be associated with white coat colour phenotypes or leucism (Reissmann & Ludwig 2013; Fleck et al. 2016). In Hereford cattle, the spotted locus was initially mapped to the KIT proto-oncogene receptor tyrosine kinase (*KIT*) gene on chromosome 6 (Grosz & MacNeil 1999). Later on indication for heterogeneity for the proportion of white coat colour in Holstein cattle was observed (Hayes et al. 2010), including association to the *KIT* and melanogenesis associated transcriptions factor (*MITF*) genes (OMIA 000209-9913). Besides other unknown genetic factors, an intronic regulatory single nucleotide variant in bovine *MITF* contributes to the differences between spotted and non-spotted phenotypes in Holstein and Simmental cattle (Fontanesi et al. 2012; Jansen et al. 2013; OMIA 000214-9913). Also in other species like dogs and horses regulatory non-coding *MITF* variants have been described to be associated with white spots on the head and the body (Hauswirth et al. 2012; Körberg et al. 2014; Negro et al. 2017). In cattle like in other species, coding variants in the *MITF* gene cause white coat colour phenotypes associated with eye malformations such as microphthalmia (Wiedemar & Drögemüller 2014; OMIA 001931-9913) or bilateral deafness (Philipp et al. 2011; OMIA 001680-9913). Variants in the human *MITF* gene are associated with Waardenburg syndrome 2A and Tietze syndrome causing deafness due to a lack of melanocytes in the inner ears and pigmentary disturbances in iridis, hair and skin (Liu et al. 1995; Shibahara et al. 2001; Grill et al. 2013; OMIM156845).

In Switzerland the so called Original Braunvieh cattle population is ancestral to the worldwide known Brown Swiss population, which was formed in the USA from animals that were obtained in Switzerland between 1869 and 1910 (Hagger 2005).

Original Braunvieh cattle have a solid brown coloured coat with a light stripe around the muzzle and black claws. In the Original Braunvieh cattle breed, two rarely occurring dominant inherited coat colour variations are known as colour-sided (Durkin et al. 2012; OMIA 001576-9913) and belted (Drögemüller et al. 2009; Awasthi Mishra et al. 2017; OMIA 001469-9913). With the introduction of artificial insemination in the 1960's, the Original Braunvieh population was introgressed with Brown Swiss individuals from North America resulting in today's Braunvieh population that represents one of the two main dairy breeds in Switzerland. The use of American Brown Swiss sires in Braunvieh cattle is still common and thus leads to Braunvieh animals with various levels of Brown Swiss genes (Stergiadis et al. 2015.) In recent years, more frequently Brown Swiss cattle with white spots on the abdomen and/or on the head (Figure 1) have been reported to the national breeding association. These white spotted animals, although accepted in the herd book, do not comply with the official breed standards and are therefore not desired. The aim of this study was to understand the molecular genetic cause for this unusual coat colour variation in Brown Swiss cattle.

Material and Methods

A total of 60 Brown Swiss animals with white spotted coat were sampled as cases. First of all, we genotyped all animals for the two recently described DNA variants associated with depigmentation phenotypes in Brown Swiss cattle: the colour-sided-associated *KIT* variant by direct PCR testing as described before (Durkin et al. 2012), and the belt-associated variant on chromosome 3 by indirect haplotype estimation (Awasthi et al. 2017). A total of 1639 animals (20 cases and 1619 controls) have been genotyped for 40'636 SNPs and were used for GWAS as described in the supplementary data. Genotyping data can be retrieved at

https://www.animalgenome.org/share/tmp/KIG1531815029.zip.gz. After whole genome sequencing of one case and subsequent variant filtering against 26 genomes of solid coloured cattle , including 11 Original Braunvieh, a total of 57 private variants remained. We then filtered these variants against the current variant repository of 2332 sequenced cattle of the 1000 bull genome project. The finally remaining two private variants (rs722765315 and rs719139527) were genotyped in all animals (Table S1). The genome data corresponding to roughly 20x coverage of the genome was made freely available under study accession no. PRJEB18113 at the European Nucleotide Archive (sample accession SAMEA19313668). Additional details on the methodology are provided in the supplementary data.

In addition, we checked if the two private variants are situated in highly conserved sequence domains with the PhastCons tool (Margulies et al. 2003) and searched also for regulatory elements from the ENCODE data at the UCSC Genome Browser (https://genome.ucsc.edu/).

Results and Discussion

Among the sampled Brown Swiss cattle collected for this study the quantity and quality of abdominal spots, facial marking and depigmented claws varied significantly (Figure S1). Visible eye abnormalities or signs of deafness in animals with white spotted coat have not been observed or reported. A single animal with white spotted coat was heterozygous for the colour-sided-associated *Cs6 KIT* variant (Durkin et al. 2012; Figure S2), whereas the belt associated structural variant in the 5'-flanking region of the *TWIST2* gene (Awasthi et al. 2017) was absent from all white spotted animals in this study. Analysing the pedigree data of the sampled cattle, we identified a common ancestor for all cases born in the year 1959 (Figure S3).

We performed a genome-wide association study (GWAS) with 20 Brown Swiss cattle with white spotted coat and 1619 Brown Swiss controls. The most significant

association was located on chromosome 22 about 612kb upstream of the *MITF* gene (Figure 2, Table S2). The marker with the strongest association was ARSBFGLNGS21229 with a p-value of 3.35×10^{-38} at chromosome 22 position 32,573,751 (Bos_taurus_UMD_3.1.1). Further significantly associated markers were found distributed over almost the entire chromosome 19 and on chromosome 6, 35 Mb away from the *KIT* gene (Figure 2; Table S2). We could not identify plausible functional candidate genes close to any of these markers. We therefore assumed that the associated SNPs on chromosomes 6 and 19 either represent false positive, spurious association signals or indicate genetic heterogeneity.

We sequenced the genome of a Brown Swiss animal with white spotting coat colour phenotype and detected a total of 2005 variants in a region of 1.3 Mb spanning the *MITF* gene on chromosome 22. We analysed the interval from the end of the *FOXP1* gene, located upstream of *MITF,* to the beginning of the *FRMD4B* gene, the downstream neighbouring gene of *MITF*. None of these variants affected the protein coding region of *MITF*. Visual inspection of the bam-files did not reveal any indications for the presence of structural variants. Variant filtering identified two private variants (g.31,908,435G>A (rs722765315), and g.32,054,240T>A (rs719139527)) located in the 5'-region of the *MITF* gene which occurred exclusively in 18 Brown Swiss and 2 Danish Red cattle (Table S3). As the Danish Red breed was introgressed with Brown Swiss individuals (Sørensen et al. 2005), these two variants are most likely private to Brown Swiss cattle and their crossbred offspring. Subsequently, we genotyped these two variants in 172 animals and observed perfect linkage disequilibrium between the two variants (Table 1). Interestingly, the single animal carrying the colour-sided-associated *Cs6 KIT* variant was also heterozygous for the two *MITF* variants (Table S4). There was a highly significant difference in *MITF* allele frequencies between Brown Swiss cattle with white spotted coat and

controls using a standard chi-square test (Table 1). The presence of homo- and heterozygotes in the cohort of white spotted animals supports a dominant mode of inheritance. However, approximately 51% (35/68) of solid coloured cattle were either homo- or heterozygous for the mutant alleles, indicating reduced penetrance. Interestingly, there is no perfect correlation between the phenotype and the genotype at the two identified *MITF* variants (Table 1; Table S4). We observed heterozygous animals that had a similar proportion of depigmented coat as homozygous animals. Extended white spots on the abdomen, the head and the legs were seen in heterozygous and in homozygous mutant cattle (Figure 3). On the other hand, some homozygous animals were found, which showed only a little white spot on the head. The white spotting was not always left/right symmetrical and our analysis was hampered by the fact that for some animals we had only one photo showing one side. The varying degrees of white spotting in selected homo- and heterozygous mutant animals are illustrated in Figure 3. Furthermore, three out of the 60 sampled Brown Swiss animals with white spotted coat were genotyped homozygous wild type at the two identified *MITF* variants (Table 1). These animals were closely related to each other (paternal half-sibs) and showed a range of white spots of varying size on the ventral abdomen. As they could not be explained by the presence of the two *MITF* variants seen in all other cases we postulate that further genetic heterogeneity contributes to white spotting phenotypes in Brown Swiss cattle. This hypothetical heterogeneity might be allelic or it might involve other coat colour loci. During genotyping of control animals we identified four Brown Swiss sires as heterozygous for the two identified *MITF* variants which showed no genealogical relationship to the abovementioned sire from 1959 (shown in Figure S3). Therefore we conclude, that the mutation event has probably occurred earlier than 1959. We could not identify a potential founder animal due to missing records of that time.

Comparative *in silico* sequence analysis showed that both variants affect highly conserved nucleotide positions (Figure 4). In humans, rodents, horses, dogs, cats, other ruminants and further diverse species the bovine wild type allele is present, but the PhastCons scores reported at UCSC genome browser of the corresponding human genome positions actually differ: 0.99 for human GRCh38.12 chr 3: g.69,792,335 which corresponds to bovine chr 22: g.31,908,435G>A (rs722765315), and 0 for human GRCh38.12 chr 3: g.69,630,671 which corresponds to bovine chr 22: g.32,054,240T>A (rs719139527). Furthermore, extrapolated from human ENCODE data the rs722765315 homologous region contained a DNaseI hypersensitive site and a H3K27ac cluster, which are characteristic hallmarks for transcriptionally active chromatin or enhancer elements (Figure S4; Creyghton et al. 2010; Rada-Iglesias et al. 2011). In these specific regions of the genome, chromatin has lost its condensed structure, exposing the DNA and making it accessible. These accessible chromatin zones are functionally related to transcriptional activity, since this remodelled state is necessary for the binding of proteins such as transcription factors. Therefore, we speculate that the bovine rs722765315 variant located in the 5'-flanking region of the bovine *MITF* gene might alter a putative cis-regulatory element controlling the regulation of *MITF* expression during development and thus cause the depigmentation phenotype.

In conclusion, we identified two single nucleotide variants in the non-coding 5'-region of the *MITF* gene which are in complete linkage disequilibrium and private to the Brown Swiss cattle breed. The mutant alleles are associated with variably expressed white spotting and appear to act in a dominant manner with incomplete penetrance. Based on evolutionary conservation and annotated epigenetic marks, we rate rs722765315 to be more likely to represent the true causative variant.

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Tables

Table 1 Genotypes of 172 Brown Swiss cattle at two variants (rs722765315 and rs719139527) in the 5'-region of the *MITF* gene.

(Allele frequencies using standard chi-square test: p-value = $5.175e^{-10}$. Please note that the samples were not selected randomly, most likely the statistics is biased because of the targeted search for cattle with white spotted coat.)

Phenotype	White spotted	Solid coloured	Unknown	Total
Number of animals	60	68	44	172
wt/wt	3	33	24	60
wt/var	20	13	10	43
var/var	37	22	10	69

Legends to figures

Figure 1. Coat colour phenotype of a solid coloured (A) and a Brown Swiss cow with white spotted coat (B). The region, where the most cases show a white coat colour is inguinal, on the ventral belly and lateral on the abdomen in front of the hind limbs (B). Most of the cases have also a white spot on the head. Additional, white claws at the hind limbs and a white end of the tail were rarely observed (B).

Figure 2. Genome-wide association study comparing Brown Swiss cattle with white spotted coat (n = 20) to solid coloured Brown Swiss cattle (n = 1619). (A) The green line indicates the Bonferroni corrected significance threshold for significant association (α=0.05; $-\log_{10}p = 5.88$). The red line indicates the Bonferroni corrected significance with a $-\log_{10}p = 6.58$ (α=0.01). (B) The quantile-quantile (QQ) plot shows the distribution of SNP markers under the null hypothesis and the curve in the left under edge indicates that these markers are stronger associated with the feature than it would be expected by chance.

Figure 3. Illustrative examples of the white spotted phenotype for different rs722765315 and rs719139527 genotypes showing no obvious phenotype/genotype correlation. The first and the last animal in one column are selected as examples with the smallest and the biggest white spot. The drawings are standardised in one row, but the spread of the white spot corresponds to the individual animals selected for these drawings. Different spread of white head markings in homozygous animals is shown in (A), while the differentially depigmented abdomen of homo- and heterozygous animals is shown in (B). Note the extended depigmented hind limbs and claws of two homozygous animal (C).

Figure 4. (A) The UCSC genome browser screenshot of the bovine UMD3.1.1 assembly shows the position of the two identified variants in the 5'-region of the *MITF* gene on bovine chromosome 22. (B) Sanger sequencing electropherograms showing the three observed genotypes at each variant position: rs722765315 and rs719139527. (C) Nucleotide conservation across 14 different mammalian species. Please note that the sequence conservation actually differ as the homologous sequence for rs722765315 is present in all mammalian species shown.

Supporting information

Appendix S1. Supplementary methods.

Table S1. Primer sequences of the two variants (rs722765315 and rs719139527) close to the *MITF* gene.

Table S2. GWAS results for white spotting in Brown Swiss cattle.

Table S3. Count of heterozygous and homozygous mutant animals at 57 different variants in the 1000 bull genome project. All variants, which were not Brown Swiss specific, were excluded. This left only two variants highlighted in yellow, which were exclusively present in Brown Swiss and Danish Red, which is known to have some introgression of Brown Swiss animals (Sørensen et al. 2005).

Table S4. Individual cattle samples of Brown Swiss cattle with the phenotype and their genotypes at the two variant positions near the *MITF* gene. If “no white spots are detected” is written in the coat colour phenotype, we cannot reliably exclude the presence of white spots, because we did not have pictures from all sides of the body.

Figure S1. Photos of the 20 cases used for GWAS.

Figure S2. Picture of the heterozygous *KIT*-associated colour-sided Brown Swiss animal, which was heterozygous for the two variants close to the *MITF* gene. In picture (A) we can see a greater spot on the head than usual in colour-sided cattle. Picture (B) shows white the back, which is typical for colour-sided animals.

Figure S3. Pedigree of the paternal line indicating the white spotted Brown Swiss cattle. The maternal line was omitted for clarity, however we identified a heterozygous tested carrier in every maternal line of a homozygous case. Squares represent males and circles females. Completely filled symbols represent animals

with a white spotted phenotype. Animals with no visible spots or unknown status are represented by a white symbol. The black and white symbol represents the *KIT*-associated colour-sided animal, which is also heterozygous for the *MITF* variants rs722765315 and rs719139527. The heterozygous genotype is visible as blue laboratory number and the homozygous variant is marked as red laboratory number. The whole genome sequenced animal is highlighted yellow. All animal defined as cases in the GWAS have a star. In the pedigree 107 animals with genotypes are visible. Wild type tested animals (n = 60) and five heterozygous animals are not shown in the pedigree to avoid confusion. The common ancestor born in 1959 is shown at the top. Four bulls who did not descend from this ancestor were tested heterozygous and have common ancestors born in the 1940s. So we conclude that this mutation event is older than initially suspected.

Figure S4 ENCODE data from the UCSC Genome Browser, which shows the histone H3K27ac mark in the human genome at the homologous position to rs722765315 (picture A) and rs719139527 (picture B). Note that the homologous region to rs722765315 is more conserved and has higher scores for H3K27ac and DNase I hypersensitivity than the homologous region to rs719139527. Therefore, we suggest that the first position (A) is the main variant causing white spotted coat in Brown Swiss cattle breed.