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Genomic regions and candidate genes associated with forehead whorl positioning in Thoroughbred horses

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Previous studies have examined the relationship between hair characteristics and temperament traits in various animals. A partial genetic association has been suggested in humans because whorl formation and neurological development occur simultaneously during the fetal period. In the horse industry, anecdotal evidence suggests a link between the forehead whorl position and temperament. In our previous study, the heritability of forehead whorl positioning was h^2=0.653, indicating significant genetic contributions in thoroughbreds. Therefore, in this study, we designed a genome-wide association study using 192 Thoroughbred horses to detect candidate genes associated with forehead whorl positioning. The results revealed 11 suggestive markers on chromosomes 2, 14, 15, 19, and 26, although no significant markers were found. Five genes, namely PTTG1, CCNH, RASA1, COX7C, and CLDN1, located near these markers had functions related to skin cell or hair follicle development and temperament. Therefore, these genes may be involved in the positioning of the forehead whorl in Thoroughbreds. The candidate genes identified in this study are expected to contribute to elucidating the genetic factors behind forehead whorl in horses and to help interpret the common process of whorl and temperament development.

Keywords: *forehead whorl, genome-wide association study, heritability, temperament, Thoroughbred horse*

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Numerous studies have investigated relationships between coat color, hair characteristics, including whorl patterns, and temperament or personality traits across various animal species, including humans. Human studies suggest that fetal hair patterning and neurological development of the brain occur in parallel during weeks 10–16 in the maternal uterus and that these processes are intrinsically related [34, 35]. Furthermore, among the hair characteristics, hair whorls are derived from the same cell layer as the fetal nervous system [35]. Several studies have reported that whorl pattern formation is determined at or just before the 17- to 25-day nervous system developmental event in utero [8] and that the pattern is related to handedness [18]. Evidence suggests that the hair whorl pattern formation and brain neurological development are controlled by a common genetic mechanism.

In horses, hair whorls on the forehead are a universally present feature suitable for objective observation, but their morphology differs among individuals [4]. Therefore, the relationships between forehead whorl pattern, behavior, and neurological function have been studied in depth [10, 28, 33]. Anecdotal folktales suggest a link between the position and number of forehead whorls and temperament traits [30]. Regarding horse personality, related genes have been comprehensively explored using genome-wide association studies (GWAS) and gene expression analyses using brain tissue [14, 36]. In addition, studies have shown candidate genes associated with personalities in humans and horses [15, 26, 48]. Furthermore, several studies using heritability calculations have reported that the position of the forehead whorl is strongly influenced by genetic factors [6, 9]. In

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the Japanese Thoroughbred population, the position of the forehead whorl showed a strong genetic influence, with h^2=0.643 [47]. However, no study has identified the genes associated with forehead whorl positioning. The only GWAS for the position of the forehead whorl was conducted on 342 Quarter Horses, which identified the associated genomic region [21].

Thoroughbred horses generally tend to have low genetic diversity [13]. The genetic structure of the Japanese Thoroughbred population is characterized by the widespread presence of single nucleotide polymorphisms (SNPs) in strong linkage disequilibrium relationships [40]. This closed population is useful for characterizing related genes in the genomic analysis of common traits. Here, we conducted a GWAS in a Japanese Thoroughbred population to search for genes involved in forehead whorl positioning.

Materials and Methods

Ethics

This study was conducted as an animal experiment under approval number AP21BRS075-1 after review by the Animal Experiment Committee of the College of Bioresource Sciences at Nihon University. The animals were kept under appropriate conditions at riding clubs or in stables at a horserace track. Blood sampling was performed by veterinarians who provided routine health care. An additional experimental volume was collected when blood was drawn as part of the health care routine. After sample collection, the animals were promptly returned to normal care.

Animals

This study included 192 Thoroughbred horses (107 males and 85 females) born between 1992 and 2020. They were either riding horses bred in Japanese riding clubs or active racehorses bred in stables at horserace tracks. The sample population did not include full siblings, and the pedigree structure consisted of 169 paternal horses and 192 maternal horses. Forty-six individuals were paternal half-siblings.

The positions of the forehead whorl, pedigree, year of birth, and sex were obtained from health passports. The information was double-checked using the passports and the Japan Bloodstock Information System. The positions were classified as high, medium, or low, according to international evaluation standards. The sample population consisted of 96 animals with the high position as controls and 96 animals with the medium and low position as cases. Because the number of horses with a low position for hair whorls was small among the sampled population, they were treated as a case group together with those with a medium position.

DNA extraction and genotyping

Genomic DNA was extracted from the collected blood samples using a NucleoSpin® Blood kit (Macherey-Nagel, Dueren, Germany) according to the manufacturer's instructions. DNA solutions were adjusted to 50–60 ng/μl using a Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) based on Qubit™ dsDNA Quantification Assay Kits (Thermo Fisher Scientific). Genotyping was performed using 192 extracted genomic DNAs and the Infinium HD iSelect Custom BC Neogen Equine Community Array (65,157 SNPs, Illumina, San Diego, CA, USA) and iScan™ optical imaging system (Illumina) according to the manufacturer's instructions. Genotypes were obtained from the scan data using the GenomeStudio software (version 2.0; Illumina). EquCab3.0 was referred to for genotype data.

Quality control

The ped and map files were output using the PLINK Input Report Plug-in v2.1.4 plug-in function of GenomeStudio software 2.0. To improve the reliability of the genotype data, quality control was performed using PLINK v1.90 with the following commands:

/plink --bfile filename --horse --mind 0.05 --geno 0.01 --maf 0.1 --hwe 0.000001 --out filename

The --mind option excludes samples where more than 5% of individuals were not genotyped. The --geno option excludes SNPs for which more than 1% were not genotyped in the study population. The --maf option excludes SNPs with minor allele frequencies of 10% or less. In addition, filtering with the --hwe option excludes SNPs with a P-value$<1.0 \times 10^{-6}$ in the Hardy-Weinberg equilibrium test. Ultimately, 60,505 SNPs were used for GWAS with 96 controls and 94 cases, omitting 4,652 SNPs and 2 cases for quality control.

GWAS between forehead whorl positions and SNPs

The GWAS was performed using PLINK v1.90. Because Thoroughbreds are a closed population and a more complex population structure is presumed, the case-control (CC) test and the Cochran-Mantel-Haenszel (CMH) test were used in this study. The cluster option was used for stratification. The "cc" flag was specified to prevent bias in each stratum. To avoid an increase in the false-positive rate due to stratification, the --ppc option was used to specify a false-positive rate of 0.01 for each stratum after stratification. Model analysis (--model) was performed using two flags, "dom" and "rec". Considering that the study included two groups, the dominant model and recessive model were used to more adequately detect genes involved in forehead whorl positioning than the CC test [22]. Each analysis was combined with a permutation analysis using the --perm option to obtain empirical P-values because observed P-values may

suffer from multiple testing. A GWAS of 118 Thoroughbreds for racing distance aptitude detected SNP markers located in the neighborhood of a single responsible gene, *MSTN*, with a suggestive *P*-value of 6.96×10^{-6} [12]. Taking account of the sample size, the significance level was set at 1.0×10^{-7} as a more stringent value in this study [32]. A retrospective GWAS on the human body mass index reported that although setting a suggestive *P*-value range of 5×10^{-8} to 5×10^{-4} would increase the number of false positives, true-positive markers would be detected [11]. Considering that the sample size of this study was smaller than that of the human GWAS, the suggestive level was set at 1×10^{-4} in this study.

Exploring the neighboring SNPs of the detected markers

Because the locational information of the SNPs identified in the GWAS was based on EquCab2.0, positions were converted to EquCab3.0, using UCSC LiftOver. SNPs in complete linkage disequilibrium within 2 Mb of the converted SNP locus were extracted from a Thoroughbred genome-wide variant database [42, 43].

Identifying candidate genes

Genes near SNPs in complete linkage disequilibrium were searched for trait-associated candidate genes. Neighboring SNPs were plotted using the NCBI Genome Viewer to identify genes within 2 Mb, ranging from SNPs at both ends. The genes were obtained from the NCBI Equus caballus Annotation Release 103 (https://www.ncbi.nlm.nih.gov/refseq/annotation_euk/Equus_caballus/103/) and Ensembl Release 111 databases (https://rapid.ensembl.org/index.html). NCBI information was preferentially selected for genes that overlapped between the two databases. These genes were selected as candidate genes, and their annotations were confirmed using the NCBI database.

Results

Related SNP markers detected in each analysis

No SNPs with a significant *P*-value (1.0×10^{-7}) were detected in any analyses. However, SNPs with a suggestive *P*-value (1.0×10^{-4}) were detected in each analysis. In the CC test, three SNPs located on chr19:30276983, chr26:8223184, and chr26:18650757 were suggestive (Fig. 1A). The study population was stratified into 25 clusters, with each cluster containing at least one individual from the high position group of forehead whorls. Five SNPs were detected in the CMH test, with the top three identical to those in the CC test and two other markers at chr14:18507899 and chr14:18612427 (Fig. 1B). Three markers located on chr2:16372239, chr2:16373122, and chr15:4740261 were detected in the dominant model analysis (Fig. 1C). Among them, chr2:16372239 and chr2:16373122 were located close to each other at a distance of 882 bp. Three markers located on chr14:80018388, chr14:80018698, and chr14:80021488 were detected in the recessive model analysis (Fig. 1D). Among them, chr14:80018388 and chr14:80018698 were located close to each other at a distance of 309 bp. A permutation analysis was performed for each of the above four analyses, and all empirical *P*-values were statistically significant ($P<0.05$).

Identified candidate genes

A single search window was defined when suggestive SNP markers located in the vicinity of each other were detected in each analysis: window 1, chr14:18507899 and chr14:18612427; window 2, chr14:80018388, chr14:80018698, and chr14:80021488; and window 3, chr2:16372239 and chr2:16373122. Other markers had independent search ranges, resulting in seven search windows (Table 1). In summary, 17, 11, 6, 3, and 4 candidate genes were found in windows 1, 2, 3, 5, and 7, respectively. Although one gene was found in each of windows 4 and 6, no functional annotations were identified.

Discussion

This GWAS identified candidate genes involved in determining the position of the forehead whorl in a Japanese Thoroughbred horse population. In our previous study, a heritability of h^2=0.643 was calculated for the position of the forehead whorl in a Japanese Thoroughbred population, indicating that the position of the forehead whorl is largely determined by genetics [47]. Genetic studies on racing distance aptitude have calculated heritability as high as h^2=0.94 [45]. Subsequently, GWAS studies have revealed that the myostatin (*MSTN*) gene is related to racing distance aptitude, and sequence and functional analyses have elucidated its contribution mechanisms [12, 29, 41]. However, only suggestive SNPs were detected in this study. These results suggest that the candidate genes associated with the positioning of forehead whorls may have minor genetic effects. In addition, while significant *P*-values were observed at specific loci in a study using Quarter Horses [21], the candidate genomic region affecting the positions of the whorls elucidated in the study was different from the region discovered in the present study using Thoroughbreds. These results may be related to the effect of sample size on the power of the analysis [16]. Furthermore, the difference might be affected by other factors, such as horse breed and rearing environment differences [25]. With regard to breeds in particular, high values have been calculated for Konic horses and Pura Raza Española horses in heritability studies of forehead whorls [6, 9]. Therefore, genomic analyses in

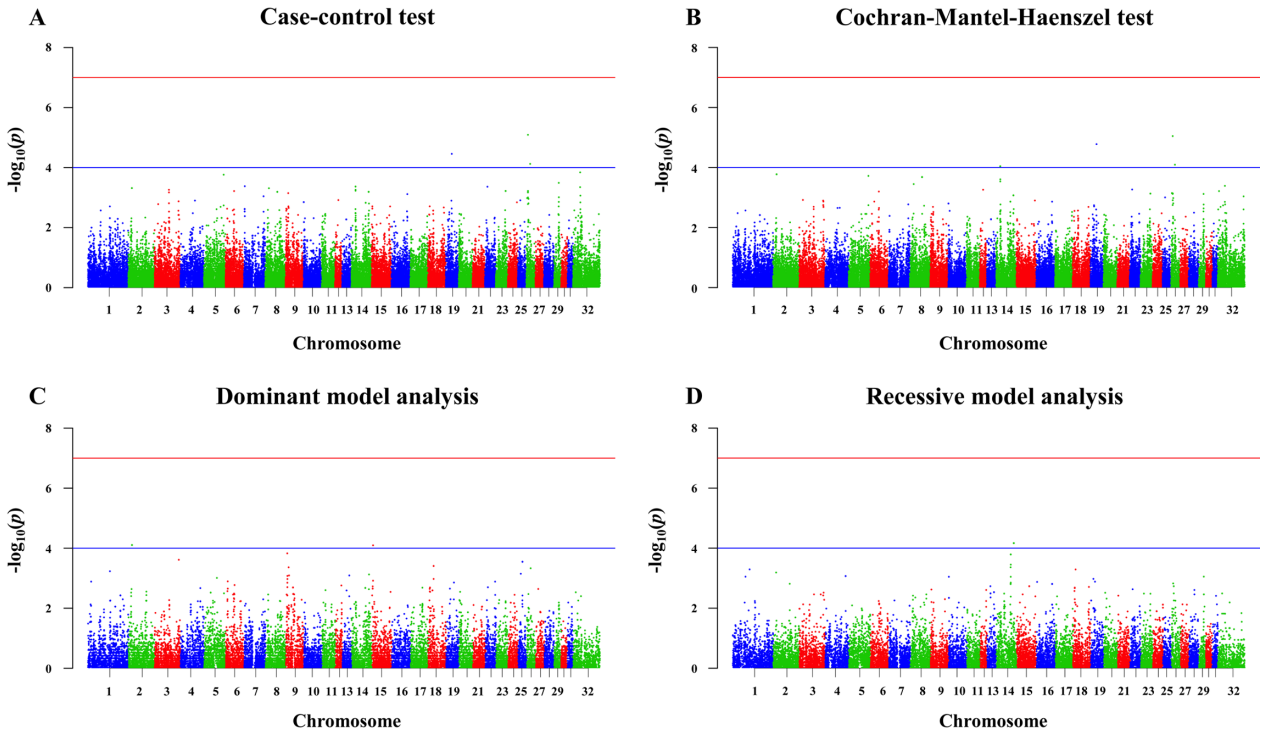

Fig. 1. Manhattan plot of forehead whorl positioning.
The x-axis indicates the number of chromosomes. The y-axis indicates the common logarithmic scale of *p*-values for each SNP. The genome-wide significance level is −log10 (1.00e-7) and indicated by a red line, and the suggestive level is −log10 (1.00e-4) and indicated by a blue line.
A shows the results of the case-control test. Three markers located at chr19:30276983, chr26:8223184, and chr26:18650757 are shown between the two lines.
B shows the results of the Cochran-Mantel-Haenszel test. Five markers located at chr19:30276983, chr26:8223184, chr26:18650757, chr14:18507899, and chr14:18612427 are shown between the two lines.
C shows the results of the dominant model analysis. Three markers located at chr2:16372239, chr2:16373122, and chr15:4740261 are shown between the two lines.
D shows the results of the recessive model analysis. Three markers located at chr14:80018388, chr14:80018698, and chr14:80021488 are shown between the two lines.

other breeds and different Thoroughbred populations are expected to provide comprehensive findings and detect a comprehensive set of genes responsible for the complex traits of forehead whorls.

Among the candidate genes detected in window 1, *PTTG1* is involved in cell polarization and migration [27], and its knockdown *in vitro* can reduce dermal fibroblast activity [46]. Furthermore, *PTTG1* is also involved in the cell cycle and regulates skin cell proliferation [2]. This suggests that changes in cell proliferation and migration patterns caused by the gene may affect the positions of forehead whorls. *Pttg1* in mice plays a role in memory consolidation and formation [23], and it may also be involved in behavior and temperament.

Among the candidate genes detected in window 2, *CCNH*, *RASA1*, and *LOC100630549* (*COX7C*) may be associated with hair follicles, and *CCNH* is a cyclin-dependent kinase regulator involved in several processes of cyclin activation and transcriptional regulation. In mice, cyclin-dependent kinase (CDK) 4 is involved in determining the number of stem cells by regulating the balance between hair follicle replication and amplification of hair follicles [17]. This suggests that CCNH, part of the CDK-activated kinase complex that activates CDK4, is involved in the cell proliferative function of hair follicles [32, 39]. *CCNA*, which encodes a cadherin protein, was identified as a candidate gene related to forehead whorl positioning in a previous study [21], suggesting that *CCNH* is a more robust candidate as a follicle-associated gene. *RASA1* encodes a protein that represses RAS. *RASA1* mutations in humans cause hair follicle malformations with anopia and affect hair follicle growth and the cell cycle [24]. In addition, studies on the development of brush hair traits in goats have reported that MAP3K1 levels are upregulated by miR-31-5p, which targets RASA1, significantly improves cell proliferation, and enhances hair quality [7]. *COX7C*

Table 1. Detected candidate genes in each marker window

Window	Chromosome	SNP marker location	Number of flanking SNPs	Association analysis*	Candidate gene**
Window 1	14	18507899 18612427	34	CMH test	*MIR9155* *LOC102148137* *MIR146A* *PTTG1* *ZBED* *LOC111767742* *LOC111767743* *CCNJL* *FABP6* *LOC111767744* *LOC111767745* *PWWP2A* *TTC1* *ENSECAG00000032213* *ENSECAG00000013696* (*SLU7*) *ENSECAG00000020786* (*C1QTNF2*) *ENSECAG00000031871*
Window 2	14	80018388 80018698 80021488	95	RMA	*CCNH* *RASA1* *LOC100630549*
					LOC111767989 *LOC100073243* *LOC100073245* *ENSECAG00000043751* *ENSECAG00000038873* *ENSECAG00000029068* *ENSECAG00000041212* *ENSECAG00000030211*
Window 3	2	16372239 16373122	15	DMA	*GUCA2B* *LOC111770286* *LOC106782782* *HIVEP3* *ENSECAG00000034425* *ENSECAG00000032365*
Window 4	26	8223184	24	CC test CMH test	*ENSECAG00000036005*
Window 5	19	30276983	195	CC test CMH test	*CLDN1* *CLDN16* *TMEM207*
Window 6	26	18650757	61	CC test CMH test	*LOC100067458*
Window 7	15	4740261	52	DMA	*LOC111768021* *LOC106781673* *MRPS9* *ENSECAG00000035348*

*CMH: Cochran-Mantel-Haenszel, RMA: recessive model analysis, DMA: Dominant model analysis, CC: case-control.
**In cases where the gene was explored only in Ensembl release 111, the genes were described with the Ensembl gene identifier.

encodes a COX subunit that catalyzes electron transfer from reduced cytochrome c to oxygen. In humans and various animal species, COX-dependent coat color affects keratinocyte differentiation, follicle development, and hair growth [19], suggesting that this effect is involved in the position of the forehead whorl. Furthermore, *COX7C* is associated with reduced mitochondrial activity in humans [1] and has been suggested to be a potential marker of Alzheimer's [44].

Several studies suggest that the disease is related to personality facets, such as neuroticism, openness, and extraversion [3, 20]. These personalities are affected by polygenetic factors [31]. Therefore, *COX7C* may be associated with temperament and behavior in horses.

Among the candidate genes detected in window 5, *CLDN1* encodes the claudin 1 protein, which forms tight junctions around cells and acts as a physical barrier. In infant mice, *Cldn1* regulates hair retention by maintaining the layered structure of hair follicles [37]. In humans, *CLDN1* is involved in the intercellular contact between keratinocytes in the epidermis [38]. Therefore, in horses, *CLDN1* may be involved in the positioning of forehead whorls by regulating the laminar structure. This gene has also been listed as a candidate gene in a GWAS for schizophrenia [49]. *TMEM207* is currently predicted to be a gene encoding an essential component of the plasma membrane. The *TMEM208* mutation in flies has been confirmed to alter cell polarity and affect hair quality and the nervous system [5]. Therefore, *TMEM207*, a gene similar to *TMEM208*, may be associated with nervous function as well.

PTTG1, *COX7C*, and *CLDN1* may be related to temperament; in particular, *PTTG1* and *CLDN1* are strongly related to cell proliferation and distribution, suggesting that they affect the concurrent development of whorl patterning in the fetal period and neurogenesis [8, 18, 34, 35]. However, this study detected none of the nine candidate genes listed as leading genes for personality and temperament in horses [26]. Therefore, to clarify this correlation, careful observation of the contribution of common genes to each trait is needed, along with the development of studies on both personality-related genes and genes that determine the position of the forehead whorl in horses.

In conclusion, the present study identified candidate genomic regions and candidate genes that may be involved in determining the positions of forehead whorls in Thoroughbred horses. Since these candidate genes are located in different genomic regions from those identified in Quarter Horses [21], genomic analyses are needed in various horse populations bred in different regions to comprehensively identify the causative genes. Further detailed analysis of the candidate genes detected in this analysis by fine mapping is expected to provide insight into the true relevance of the genes involved in forehead whorl positioning. The identification of candidate genes associated with forehead whorl positioning may be useful for understanding the partial genetic background of the personalities and temperaments of horses.

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