Journal of Animal and Veterinary Advances 10 (14): 1855-1858, 2011
ISSN: 1680-5593
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Polymorphism Analysis of the Horse Dopamine Receptor D4 Gene *(DRD4)* Sequence

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Abstract: In this study, partial sequence of horse *DRD4* gene was cloned including intron 1, partial exon 1 and exon 2. And restriction endonuclease Stu I was used to analyze the polymorphism of the *DRD4* gene sequences of 270 horses from six types including importing breed, cultivating breed and local breed. The products of endonuclease cutting were detected by 8% non-denatured polyacrylamide gel electrophoresis and showing in silver staining protocol.The result indicated restriction endonuclease Stu I showed polymorphism. Six kinds of genotypes were found in six populations which were controlled by three alleles. The results of Chi-square (χ^2) test showed that genotypes of horse *DRD4* gene in TB, SH, XN did not fit with Hardy-Weinberg equilibrium ($p<0.05$) but in WS, BH and WZ fit with Hardy-Weinberg equilibrium ($p>0.05$).

Key words: Horses, *DRD4* gene, polymorphism, PCR-RFLP, silver staining, local bread, China

INTRODUCTION

Dopamine D4 Receptor (DRD4) is one of five member receptors known to function in mammalian dopaminergic pathways. *DRD4* gene includes genetics order in junction and thus can control the brain to form ability of a certain receptor. These receptors are distributed on the surface of neuronal cells and can bind dopamine. It is well known that dopamine is a predominant catecholamine neurotransmitter in the mammalian brain where it controls a variety of functions including locomotor activity, cognition and emotion (Missale *et al*., 1998). In particular, *DRD4* is the 1st gene that has been found to affect personality. For example, there is connection between the number of 48 base pair repeats in exon and Novelty Seeking (NS) personality trait (Benjamin *et al*., 1996).

The polymorphism of the DRD4 exon region has been reported in other mammalian species such as non-human primates, dogs and horses (Livak *et al*., 1995; Niimi *et al*., 1999, 2001; Hasegawa *et al*., 2002; Ito *et al*., 2004). The genetic polymorphism is proposed to be associated with human personality traits (Benjamin *et al*., 1996; Ebstein *et al*., 1996). In addition, polymorphisms have been discovered other regions of human and dog *DRD4* gene which are thought to be associated with personality traits. The horse is one of the oldest domestic species. In the past, horse has been used so widely as communication, transport, agriculture progress and so on. With the advent of science and technology however, the horse has lost its original use and is used primarily for recreational purposes today. Thus, improving breed characteristics for recreation purposes, i.e., selecting for personality traits will add tremendous economic value to existing breeds. The potential role of human DRD4 in personality traits has attracted considerable attention in recent years but not in horses. There are few studies on VNTR of *DRD4* gene exon III related to NS behavior in horses but other regions of horse *DRD4* gene have not been studied. The objective of this study was to analyze polymorphisms of the partial *DRD4* gene sequence among six types of horse.

MATERIALS AND METHODS

The peripheral blood samples of the Thoroughbred (n = 50) were collected at the Huajun Stud Stable in Beijing. Blood samples were taken from Sanhe (n = 51), Xinihe (n = 45) and Barhu (Baerhu) (n = 54) horses in Hulunboir Aimag (Hulunbeier prefecture), Northeast of Inner Mongolia. Blood samples were collected from the Ujimchin (Wuzhumuqin) horse (n = 40) in Shilingol Aimag (Xilinguole prefecture), Central Inner Mongolia whereas blood samples of Wushen horse (n = 30) were collected at Ordos area in western Inner Mongolia. Genomic DNA was isolated from each peripheral blood sample by using the phenol-choroform and proteinase K method. The

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DRD4 genotypes were analyzed using the PCR-RFLP method. The primers were designed based on the nucleotide sequence of human, mouse and ferret *DRD4* gene. 5FA:

Forward 5'-CTGCAGACGCCCACCAACT-3'
Reverse 5'-TGGCGCACAGGTTGAAGAT-3'

A 50 ng of template genomic DNA, 18 pmoles of each primer, 7.5 μL 2×GC buffer II, 2.4 μL dNTP (2.5 Mm each) and 0.75 units of LA Taq polymerase (Takara) were mixed in 15 μL of reaction mixture. After initial incubation at 94°C for 5 min, PCR amplification was performed for 35 cycles composed of denaturation at 94°C for 40 sec, annealing for 40 sec and extension at 72 °C for 40 sec~1 min. This was followed by a final extension at 72°C for 7 min. The PCR products were analyzed by electrophoresis in 1.2% agarose gel. The PCR products were digested with Stu I enzyme. Digestion products were separated electrophoretically in 8% nondenatured polyacrylamide gel electrophoresis (Acr/Bis = 29:1, 200 v, 1.5~2.0 h) and showing in silver staining protocol. Frequencies of distribution of alleles within the herds were compared with Chi-square (χ^2) test.

RESULTS AND DISCUSSION

Through primer pair 5FA, successfully amplified out the expected fragment containing intron I, partial exon I and II region of *DRD4* gene. The electrophoresis profiles are shown in Fig. 1. After PCR fragments were detection by PCR-RFLP method and sequencing, one SNP and one inserts/deletion were identified in intron I. Genotypes and allele of the *DRD4* gene are shown in Fig. 2. For the polymorphism, six genotypes and three alleles were distinguishable according to their restriction fragment lengths: 316, 324 and 1121 bp (A allele); 333, 324 and 1121 bp (B allele); 640 and 1121 bp (C allele). By comparing the sequences of two types homozygote on SNP found that two sites showed nucleotide transition from A-G and insert/delete from 17 bps.

Statistical analysis of the site showed that dominant genotype and alleles of Thoroughbred are AB and B and dominant genotype and alleles of other breeds horse are AA and A. The results of Chi-square (χ^2) test showed that genotypes of *DRD4* gene in TB, XN and SH did not fit with Hardy-Weinberg equilibrium ($p<0.01$) but in BH, WZ and WS, they did fit with Hardy-Weinberg equilibrium ($p>0.05$) (Table 1).

According to the breed history, the Sanhe horse is a locally-selected breed whereas the Thoroughbred is an imported breed. Their offspring breeding is mostly by

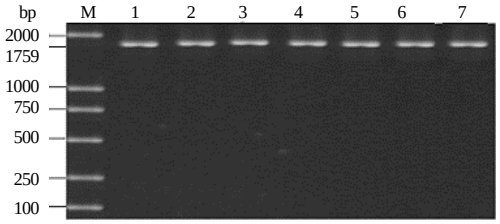

Fig. 1: Gel diagram of prolactin fragment amplified by primer pair 5FA before digestion

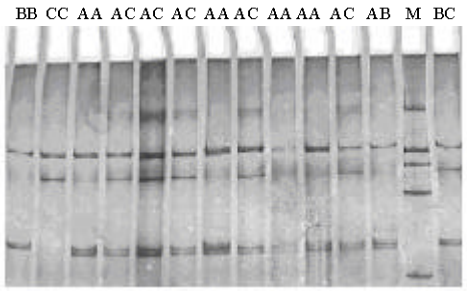

Fig. 2: Restriction analysis of DRD4 1759 bp PCR products digested with Stu I by 8% nondenatured polyacrylamide gel electrophoresis stained with silver M.DL2000 marker; BB genotype = 333, 324 and 1121 bp; CC genotype = 640 and 1121 bp; AA genotype = 316, 324 bp, 1.121 kb; AC genotype = 316 and 324 bp, 1.121 kb, 639 bp; AB genotype = 316, 324 and 333 bp, 1.121 kb; BC genotype = 333 and 324 bp, 1.121 kb, 639 bp

artificial selection. The artificial selection of offspring breeding is more rigorous in Thoroughbred than in others. This leads to disequilibrium of gene distribution. WS, XN, WZ and BH belong to different local populations of the Mongolian horse. These populations were bred in a relatively isolated natural environment.

Thus, the distribution of genes in these horses is close to equilibrium. The Xinihe horse is a local breed which is originated from the Northeastern Inner Mongolia (Hulunboir Aimag). The region is famous for horses for their speed and stamina because of a long history of breeding for races, including introduction of horses from other regions. Offspring breeding of Xinihe horse has artificial selection of a bigger ratio, so disequilibrium of gene distribution can also appear (Lai, 2003).

One SNPs (1444G/A) and one insert/delete of the region have been found by polymorphism analysis of the sequence. These variations exist in Intron which do not lead to variation in amino acids. In humans, the variations in the intron regions are known to be associated with personality traits or psychiatric disorders such as the

Table 1: Genotype distribution and allele frequencies at Stu I site of different breeds horse

		Genotypes frequency						Allele frequency			
Breed	Sample size	AA	BB	CC	AB	AC	BC	A	B	C	(χ^2) (df)
XN	45	0.444	0.133	0.044	0.156	0.200	0.022	0.622	0.222	0.156	11.39*
		(20)	(6)	(2)	(7)	(9)	(1)				
BH	54	0.370	0.130	0.056	0.296	0.130	0.019	0.583	0.287	0.130	9.11
		(20)	(7)	(3)	(16)	(7)	(1)				
WZ	40	0.575	0.025	0.000	0.175	0.175	0.050	0.750	0.138	0.112	1.26
		(23)	(1)	(0)	(7)	(7)	(2)				
WS	30	0.533	0.000	0.067	0.033	0.333	0.033	0.717	0.033	0.250	0.75
		(16)	(0)	(2)	(1)	(10)	(1)				
SH	51	0.392	0.176	0.078	0.255	0.059	0.039	0.549	0.324	0.127	21.05**
		(20)	(9)	(4)	(13)	(3)	(2)				
TB	50	0.040	0.280	0.100	0.420	0.020	0.140	0.260	0.560	0.180	14.48**
		(2)	(14)	(5)	(21)	(1)	(7)				

d: df = 5, χ^2 (0.05) = 11.07, χ^2 (0.01) = 15.09. *Means significant difference (0. 01<p<0.05), **Means most significant difference (p<0.01)

association of the tryptophan hydroxylase gene intron VII with aggression in Schizophrenia and Schizoaffective disorders and the association of the serotonin transporter gene (5HHT) intron II with anxiety (Hong *et al*., 2001; Melke *et al*., 2001). In the case of DRD4, variation of a variable number of repeated G nucleotides in the intron I region has been reported although, it does not contribute to the susceptibility to Schizophrenia (Barr *et al*., 1993). In the neurotransmitter related genes, variation of a variable number of tandem repeat sequence such as the intron II region of human 5HTT has affected reporter gene expression (MacKenzie and Quinn, 1999). In Primates and dog, the insert/deletion polymorphisms were found in the intron II. Insert/deletion of 17 bp was found in the intron II region of dogs *DRD4* gene and inserts/deletions of 6 and 8 bp were found in intron II region of humans and apes *DRD4* gene (Shimada *et al*., 2004; Hidetoshi *et al*., 2005). The polymorphisms do not relate to reporter gene expression.

So, further studies are required to determine the effect of variation in the horse DRD4 intron I region on reporter gene expression. Other associations between intronic variation and functional differences are possible. For example, alternative splicing such as the gene of Tau, a microtubule-associated protein or linkage with an exonic variant has been reported in DRD2 (O'Hara *et al*., 1993; Spillantini *et al*., 2000). Because, variation in the horse DRD4 intron I region was not linked with previously reported Exon polymorphic regions, this region can be an independent marker for the further survey of the relationship between genotype and horse behavioral traits. It can be postulated that the association between intronic variations and differences in behavioral trait is related to a change in Transcription Factor (TF) binding sites, alternative splicing and linkage with exonic variant (O'Hara *et al*., 1993; Fiskerstrand *et al*., 1999; Spillantini *et al*., 2000). In order to elucidate the mechanism of these associations, expression analysis may be effective because variations in intron II region of humans and apes *DRD4* gene can lead to changes in TF binding sites (Shimada *et al*., 2004). In humans after *DRD4* gene was cloned in 1991, some genetic markers associated with personality were identified which have become the basis for investigating *DRD4* gene in other mammals (Van Tol *et al*., 1991).

Would there be some associations between these polymorphisms in horse *DRD4* gene and personality? It still requires more data on the sequence variations of this gene with a greater number of individuals for each breed in the future.

CONCLUSION

The present study is the 1st report to examine the polymorphisms of DRD4 in the Mongolia horse with the sequence compared with other species. We found some novel polymorphisms using RFLP among six breeds including a total of 270 individuals. Thus, the analysis of these polymorphisms in *DRD4* gene is a useful means for the studies of evolution and behavior sciences.

ACKNOWLEDGMENTS

The researchers are grateful for the support of Henan Institute of Science and Technology Startup Foundation for Doctor (No. 7014), Anhui Provincial Natural Science Foundation (No. 090411019) and the National Natural Science Foundation of China (30760162). Caiyun Fan and Jianbo Cheng contributed equally to this work.

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