犬のチロシン水酸化酵素遺伝子およびドーパミンβ水酸化酵素遺伝子

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Canine Tyrosine Hydroxylase (TH) Gene and Dopamine β-Hydroxylase (DBH) Gene: Their Sequences, Genetic Polymorphisms, and Diversities among Five Different Dog Breeds

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ABSTRACT. Dopamine and noradrenaline are catecholamine neurotransmitters that are produced by biosynthetic enzymes such as tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH). As a first step to elucidate the genetic background of canine behavioral traits, we selected these genes as targets and sequenced these canine genes, and found that both were highly homologous with those of human beings. Then brain cDNAs derived from ten unrelated Beagles were used to search for polymorphisms in these genes. Four single nucleotide polymorphisms (SNPs) (C97T, G168A, G180A and C264T), one of which (C97T) will cause amino acid substitution in the TH gene, and two SNPs (C789A and A1819G), both of which will cause amino acid substitutions in the DBH gene were identified. The allelic frequencies among five dog breeds (47 Golden Retrievers, 41 Labrador Retrievers, 40 Malteses, 26 Miniature Schnauzers, and 39 Shibas) were examined and found to have significant variation between them with regards to all these SNPs, except for C97T in the TH gene and A1819G in the DBH gene. The polymorphisms of C97T and A1819G were found only in the Shiba. The present results suggest that the polymorphisms of the genes encoding catecholamine biosynthetic enzymes may become important markers for examining the genetic background of behavioral characteristics in dogs.

KEY WORDS: breed difference, DBH, dog, polymorphism, TH.

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Since a genetic polymorphism related to the dopamine D4 receptor (DRD4) was postulated to be related to the "novelty seeking" behavior [2, 9], numerous genetic markers for such behavioral traits have been researched in the human psychiatric field [1, 5, 6, 17, 23, 30]. Most of these markers are on genes coding for neurotransmitters, neuropeptides, neurotropic factors or transcription factors, as well as their receptors, synthetic enzymes and metabolic enzymes. Such research is based on the fundamental concept that one's personality is formed from the activity and/or turnover of such components of the hypothalamo-limbic system. Although this system is thought to be conserved in mammals, few polymorphic markers that could be responsible for the behavioral traits or temperament in the other mammals have been listed up. This situation is no exception in case of dogs even though we can now access various canine genome resources for linkage analysis, i.e. the 1 Mb resolution radiation hybrid map of the canine genome [10], the map of 4249 genetic markers featuring one marker every 900kb [3]. Therefore we have tried to search genetic markers that would be related to the behavioral traits in dogs and so far found some single nucleotide polymorphisms (SNPs) in neurotransmitter-related genes [12, 19, 20].

In this study tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH) genes were selected for their possible relation to canine behavioral traits. We sequenced these genes, and searched for genetic polymorphisms in these genes. The TH and DBH are biosynthetic enzymes for the synthesis of catecholamines, such as dopamine and noradrenalin, which are important neurotransmitters for the regulation of motor coordination and emotional status (arousal) in the brain. The TH is the rate-limiting enzyme in the synthesis of catecholamines, and is responsible for the conversion of tyrosine to dopa. Polymorphisms on this gene are reported to be related to human personality [29], mood disorders [16, 18, 35], schizophrenia [22, 38] and essential hypertension [36]. The DBH converts dopamine to noradrenalin. Polymorphisms on this gene are reportedly related to the attention deficit hyperactivity disorder [8, 32], Parkinson's disease [13], and smoking [21] in human beings.

MATERIALS AND METHODS

The canine homologue sequences of TH and DBH genes were determined by RT-PCR method. The complementary DNA (cDNA) was constructed from mRNA derived from the brain of a Beagle [12, 19, 20]. Consensus primers were designed based on the previously identified sequences of human, mouse, rat and bovine TH (GenBank: NM_000360, M69200, L22651, and M36705, respectively), and human, mouse, rat, equine, and bovine DBH (GenBank: Y00096, S50200, L12407, AB029430, and AF118638, respectively). For the 5' and 3' regions, the rapid amplification of cDNA ends (RACE) methods were applied using the SMART RACE cDNA Amplification Kit (Clontech, U.S.A.; for 5' identification) and the 3'-Full RACE Core Kit (TaKaRa, Japan; for 3' identification). Nucleotide sequences of the canine TH and DBH genes were determined by the dye-ter-

mination method using a DNA sequencer (ABI PRISM 3100 Genetic Analyzer, Perkin-Elmer, U.S.A.) and submitted to GenBank.

Our strategy to find polymorphisms responsible for the individuality of behavioral traits has been to screen coding regions first as they are most likely to be related to their functions. We sequenced the mostly full length of these genes from cDNAs of 10 unrelated Beagles with succeeding primer sets (forward: 5'-CAGGCCGAAGCCATCATG-3' reverse: 5'-TATTGGGATTGTGAGTGGGC-3' for the TH gene, forward: 5'-TCTTCCTGGTCATCCTGGTG-3' reverse: 5'-TATGGCTCTTGCAGAGCTCC-3' for upstream part of the DBH gene and forward: 5'-GATGGCCAAGAGATAGAGATCG-3' reverse: 5'-CAGCACACCAACACAGATCG-3' for downstream part of the DBH gene), in order to find polymorphisms in these genes. PCR was performed with 100 *n*g of cDNA in a 100 μl reaction volume, which consisted of 200 μM of dNTPs, 10 μl 10 × Ex Taq Buffer, 0.3 μM of each primer, and 1.25 units of TaKaRa Ex Taq (TaKaRa). After an initial denaturation at 95°C for 5 min, PCR was performed using 35 successive cycles of 95°C for 60 sec, 64°C for 30 sec, and 72°C for 60 sec. Chain elongation at 72°C was extended to 10 min after the final cycle for the TH gene. PCRs for both upstream and downstream parts of the DBH gene were performed with 100 *n*g of cDNA in a 100 μl reaction volume, which consisted of 200 μM of dNTPs, 10 μl 10 × Ex Taq Buffer, 0.3 μM of each primer, and 1.25 units of TaKaRa Ex Taq. After an initial denaturation at 95°C for 5 min, PCR was performed using 35 successive cycles of 95°C for 30 sec, 54°C for 30 sec (for upstream part) or 65°C for 60 sec (for downstream part), and 72°C for 60 sec. Chain elongation at 72°C was extended to 10 min after the final cycle for the DBH gene.

In order to assess genetic variation in the putative polymorphisms, peripheral blood samples were obtained from 193 individuals of 5 dog breeds (47 Golden Retrievers, 41 Labrador Retrievers, 40 Malteses, 26 Miniature Schnauzers, and 39 Shibas) [12, 19, 20]. Genomic DNA was extracted with the QIAamp Blood Midi Kit (QIAGEN, U.S.A.), dissolved into H_2O, and stored at 4°C until PCR was performed. The methods for genotyping the identified SNPs were as follows.

TH gene C97T, G168A, G180A, and C264T: The sequence of the forward primer was 5'-AGCAGGTGCTCACAGACA-3', and the reverse primer was 5'-TGTGTGAGTCCCATGGAGA-3'. PCR was performed with 40 *n*g of genomic DNA in a 100 μl reaction volume, which consisted of 200 μM of dNTPs, 10 μl 10 × Ex Taq Buffer, 0.5 μM of each primer, and 1.25 units of TaKaRa Ex Taq. After an initial denaturation at 95°C for 5 min, PCR was performed using 35 successive cycles of 95°C for 60 sec, 60°C for 60 sec, and 72°C for 30 sec. Chain elongation at 72°C was extended to 10 min after the final cycle. The successfully amplified fragments were directly sequenced and genotyped.

DBH gene C789A: The sequence of the forward primer was 5'-GCTTCTGGCAGATTCTGTGG-3', and the reverse primer was 5'-GGCAGACTTTGAGCTCTTGG-3'. PCR was performed with 30 *n*g genomic DNA in a 100 μl reaction volume, which consisted of 200 μM of dNTPs, 10 μl of 10 × Ex Taq Buffer, 0.5 μM of each primer, and 1.25 units of TaKaRa Ex Taq. After initial denaturation at 95°C for 5 min, PCR was performed using 33 successive cycles of 95°C for 60 sec, 60°C for 30 sec, and 72°C for 30 sec. Chain elongation at 72°C was extended to 5 min after the final cycle. Genotyping was done by the restriction enzyme fragment length polymorphism (RFLP) method as follows. The PCR product (35 μl) was digested with 5 units of HpyCH4 III enzyme (NEB, U.S.A.) and 5 μl of NEB buffer 4 in a total volume of 50 μl. The products were then electrophoresed on 3.0% agarose gel in 1 × TBE to estimate the product size (CC; 361 bp, CA; 361 bp, 290 bp and 71 bp, AA; 290 bp and 71 bp).

DBH gene A1819G: The sequence of the forward primer was 5'-GGTAAATGGGATCTGCAACC-3', and the reverse primer was 5'-AGGCAGAGCTATTCCACAGG-3'. PCR was performed with 30 *n*g genomic DNA in a 100 μl reaction volume, which consisted of 200 μM of dNTPs, 10 μl of 10 × Ex Taq Buffer, 0.5 μM of each primer, and 1.25 units of TaKaRa Ex Taq. After initial denaturation at 95°C for 5 min, PCR was performed using 30 successive cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. Chain elongation at 72°C was extended to 5 min after the final cycle. Genotyping was also done by the RFLP as follows. The PCR product (30 μl) was digested with 9.6 units of Bgl I enzyme (TOYOBO, Japan) and 5 μl of 10 × H Buffer in a total volume of 50 μl. The products were then electrophoresed on 3.0% agarose gel in 1 × TBE to estimate the product size (AA: 236 bp, CA: 236 bp, 164 bp and 72 bp, AA: 164 bp and 72 bp).

In order to analyze the breed differences of identified SNPs, χ^2 tests for independence were used. If the identified SNP was not found in all 5 breeds, the statistics were done using the data from breeds with an identical SNP.

RESULTS

The genes amplified in this study contained a 1, 488 bp (TH) and 1,878 bp (DBH) open reading frame, and the complete nucleotide sequences of canine TH and DBH cDNAs were submitted to GenBank with the accession numbers of AB097058 and AB097057, respectively. The identified sequences were identical to those obtained from the canine whole genome shotgun sequence that has recently (January 2005) been made available (GenBank; NW_139885 chromosome 18 for TH gene and NW_139867 chromosome 9 for DBH gene). The respective homologies at the nucleotide level of human, mouse, rat and bovine genes were 87%, 83%, 82% and 86% (TH); 79%, 74%, 74%, and 79% (DBH); those at predicted amino acid level were 89%, 87%, 89% and 86% (TH); 74%, 70%, 70% and 75% (DBH) (Fig. 1). There is a 16 amino acids insertion in the canine DBH gene at the 527th residue from the first methionine (Fig. 1).

Fig. 1. Alignment of deduced amino acid sequences of canine TH (left panel) and DBH (right panel) and other mammalian genes. The deduced amino acid sequence of canine TH was aligned with those of human (NM_000360), mouse (M69200), rat (L22651), and bovine (M36705), and that of canine DBH was aligned with those of human (BC017174), mouse (S50200), rat (L12407), equine (AB029430) and bovine (AF118638). The SNP sites are boxed. The complete nucleotide sequences of canine TH and DBH cDNAs have been submitted to GenBank with the accession numbers of AB097058 and AB097057, respectively.

Table 1. Genotype, allele frequencies and heterozygosities of polymorphisms on TH gene in five dog breeds

Breed		Genotype			Allele		H-obs
C97T	n	CC	CT	TT	C	T	
GLD	46	46 (100.0)	0 (0.0)	0 (0.0)	92 (100.0)	0 (0.0)	–
LAB	41	41 (100.0)	0 (0.0)	0 (0.0)	82 (100.0)	0 (0.0)	–
MLT	39	39 (100.0)	0 (0.0)	0 (0.0)	78 (100.0)	0 (0.0)	–
MS	25	25 (100.0)	0 (0.0)	0 (0.0)	50 (100.0)	0 (0.0)	–
SHIBA	38	18 (47.4)	11 (28.9)	9 (23.7)	47 (61.8)	29 (38.2)	0.478
Total	189	169 (89.4)	11 (5.8)	9 (4.8)	349 (92.3)	29 (7.7)	0.142
G168A	n	GG	GA	AA	G	A	
GLD	46	44 (95.7)	1 (2.2)	1 (2.2)	89 (96.7)	3 (3.3)	0.064
LAB	41	31 (75.6)	6 (14.6)	4 (9.8)	68 (82.9)	14 (17.1)	0.287
MLT	39	38 (97.4)	0 0.0)	1 (2.6)	76 (97.4)	2 (2.6)	0.051
MS	25	25 (100.0)	0 0.0)	0 (0.0)	50 (100.0)	0 (0.0)	–
SHIBA	38	33 (86.8)	5 (13.2)	0 (0.0)	71 (93.4)	5 (6.6)	0.125
Total	189	171 (90.5)	12 (6.3)	6 (3.2)	354 (93.7)	24 (6.3)	0.119
G180A	n	GG	GA	AA	G	A	
GLD	46	43 (93.5)	0 (0.0)	3 (6.5)	86 (93.5)	6 (6.5)	0.123
LAB	41	32 (78.0)	4 (9.8)	5 (12.2)	68 (82.9)	14 (17.1)	0.287
MLT	39	36 (92.3)	2 (5.1)	1 (2.6)	74 (94.9)	4 (5.1)	0.099
MS	25	18 (72.0)	6 (24.0)	1 (4.0)	42 (84.0)	8 (16.0)	0.274
SHIBA	38	38 (100.0)	0 0.0)	0 (0.0)	76 (100.0)	0 0.0)	–
Total	189	167 (88.4)	12 (6.3)	10 (5.3)	346 (91.5)	32 (8.5)	0.155
C264T	n	CC	CT	TT	C	T	
GLD	46	40 (87.0)	1 (2.2)	5 (10.9)	81 (88.0)	11 (12.0)	0.213
LAB	41	26 (63.4)	10 (24.4)	5 (12.2)	62 (75.6)	20 (24.4)	0.373
MLT	39	36 (92.3)	2 (5.1)	1 (2.6)	74 (94.9)	4 (5.1)	0.099
MS	25	18 (72.0)	6 (24.0)	1 (4.0)	42 (84.0)	8 (16.0)	0.274
SHIBA	38	29 (76.3)	5 (13.2)	4 (10.5)	63 (82.9)	13 (17.1)	0.287
Total	189	149 (78.8)	24 (12.7)	16 (8.5)	322 (85.2)	56 (14.8)	0.253

The percentage in each category is shown in parenthesis.
H–obs; observed heterozygosity.
GLD; Golden Retriever, LAB; Labrador Retriever, MLT; Maltese, MS; Miniature Schnauzer.

Four SNPs were identified in the coding region of the TH gene: a cytosine-to-thymine substitution at the 97th nucleotide (C97T), a guanine-to-adenine substitution at the 168th nucleotide (G168A), a guanine-to-adenine substitution at the 180th nucleotide (G180A), and a cytosine-to-thymine substitution at the 264th nucleotide (C264T). The SNP of C97T will cause amino acid substitution of arginine to cystein. Two SNPs were identified in the coding region of the DBH gene: a cytosine-to-adenine substitution at the 789th nucleotide (C789A) and an adenine-to-guanine substitution at the 1819th nucleotide (A1819G), which will cause amino acid substitution of asparagine to lysine and serine to glycine, respectively. The positions of these SNPs are shown in Fig. 1.

The genotype and allele frequencies of the TH and DBH polymorphic regions in five dog breeds are presented in Tables 1 and 2. The inter-breed differences with regards to the actual number of genotypes and alleles were highly significant in the four SNPs, based on the χ^2 test (G168A; genotype: χ^2=16.6, df=6, p=0.05 and allele: χ^2=16.4, df=3, p<0.01. G180A; genotype: χ^2=16.8, df=6, p<0.01 and allele: χ^2=9.25, df=3, p<0.05. C264T; genotype: χ^2=18.8, df=8, p<0.05 and allele: χ^2=12.7, df=4, p<0.05. C789A; genotype: χ^2=81.7, df=8, p<0.01 and allele: χ^2=103.8, df=4, p<0.01). The C97T and A1819G polymorphisms were seen only in Shibas.

DISCUSSION

The amplified genes in this study seem to be the actual TH and DBH genes due to their high homology with other mammalian species (Fig. 1). There is a distinctive insertion of 16 amino acids at the 527th residue from the first methionine only in the dog's DBH gene (Fig. 1). The functional significance of this sequence and whether this insertion is canine-specific or carnivore-specific remain to be solved.

Three silent SNPs and one SNP with amino acid substitution on the TH gene and two SNPs with amino acid substitutions on the DBH gene were found in this study. Most of these SNPs were on the sites that are not conserved across species (Fig. 1). Some research has pointed out that those polymorphisms conserved across species are more likely to be involved in the regulation of vital functions [24, 27, 31]. However, this hypothesis has yet to be confirmed after all

Table 2. Genotype, allele frequencies and heterozygosities of polymorphisms on DBH gene in five dog breeds

Breed	Genotype						Allele				H-obs
C789A	n	CC		CA		AA	C		A		0
GLD	45	10	(22.2)	17	(37.8)	18 (40)	37	(41.1)	53	(58.9)	0.490
LAB	40	5	(12.5)	11	(27.5)	24 (60)	21	(26.3)	59	(73.8)	0.392
MLT	38	22	(57.9)	15	(39.5)	1 (2.6)	59	(77.6)	17	(22.4)	0.352
MS	23	17	(73.9)	5	(21.7)	1 (4.3)	39	(84.8)	7	(15.2)	0.264
SHIBA	38	31	(81.6)	7	(18.4)	0 (0.0)	69	(90.8)	7	(9.2)	0.169
Total	184	85	(46.2)	55	(29.9)	44 (23.9)	225	(61.1)	143	(38.9)	0.476
A1819G	n	AA		GA		GG	A		G		
GLD	20	20	(100.0)	0	(0.0)	0 (0.0)	40	(100.0)	0	(0.0)	–
LAB	20	20	(100.0)	0	(0.0)	0 (0.0)	40	(100.0)	0	(0.0)	–
MLT	20	20	(100.0)	0	(0.0)	0 (0.0)	40	(100.0)	0	(0.0)	–
MS	20	20	(100.0)	0	(0.0)	0 (0.0)	40	(100.0)	0	(0.0)	–
SHIBA	39	10	(25.6)	12	(30.8)	17 (43.6)	32	(41.0)	46	(59.0)	0.490
Total	119	90	(75.6)	12	(10.1)	17 (14.3)	192	(80.7)	46	(19.3)	0.313

The percentage in each category is shown in parenthesis.
H-obs; observed heterozygosity.
GLD; Golden Retriever, LAB; Labrador Retriever, MLT; Maltese, MS; Miniature Schnauzer.

SNPs are identified and analyzed for their relationship to personality and/or psychiatric disorders. The effect of silent SNPs should also be analyzed [4]. Since the locations of all SNPs identified in this study were different from those of SNPs in human TH and DBH genes, possible structural and functional changes derived from these SNPs should be examined as have been reported for human genes [7, 33, 34].

With regards to the breed differences of these SNPs, C97T on the TH gene and A1819G on the DBH gene are only found in Shibas. This may imply that Shiba is a less-domesticated canine breed with particular genetic variety based upon the finding from our study and the previous report [28] in which the Shiba was genetically categorized more closely to the wolf than any other breed analyzed in this study. The alleles of 168A and 264T on the TH gene are found in the Labrador Retriever with relatively high frequency. According to the research of Hart and Hart [11], only the Labrador Retriever ranked relatively low in 'demand for affection' and 'general activity' character traits, and higher in 'ease of housebreaking' compared to the other four breeds in this study. At G180A, the Labrador Retriever and Miniature Schnauzer have relatively more A alleles compared to the other breeds. These two breeds were shown to rank higher in 'territorial defense' in the same study. At C789A, the ratio of allele frequency of the Golden and Labrador Retrievers to other breeds is reversed. Similarly, Retrievers were shown to rank lower in 'excitability', 'watchdog barking', 'dominance over owner', 'snapping at children', 'aggression toward other dogs', 'destructiveness' and 'excessive barking', and rank higher in 'obedience trainig' compared to the other three breeds in the previous study [11]. The relationship between our study and the research about breed difference of behavioral traits should be examined in near the future.

Although the draft sequence of canine genome was unveiled on the website of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/genome/guide/dog/) on July 2004, only a few genetic markers that will be related to canine behavioral traits have been listed up [12, 14, 15, 19, 20, 25, 26, 37]. This situation is possibly a result of the lack of researchers devoted to this area of science. However, the dog is an ideal animal for this type of research, since they live in simpler social systems than humans and can express more emotions than experimental rodents, such as rat and mouse. This kind of research will lead us to reveal not only the genetic diversity of canine breeds but also the genetic background of canine behavioral traits.

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