NOTE *Ethology*

Sequences of Canine *Glutamate Decarboxylase (GAD) 1* and *GAD2* Genes, and Variation of their Genetic Polymorphisms among Five Dog Breeds

Sayaka ARATA[1)], Chie HASHIZUME[1)], Takefumi KIKUSUI[1)], Yukari TAKEUCHI[1)]* and Yuji MORI[1)]

[1)]*Laboratory of Veterinary Ethology, The University of Tokyo, Tokyo 113–8657, Japan*

(Received 3 March 2008/Accepted 20 May 2008)

ABSTRACT. Glutamate decarboxylase (GAD) is the primary enzyme in the brain that catalyzes the synthesis of γ-aminobutyric acid (GABA), the main inhibitory neurotransmitter. There are two isoforms named according to their molecular weights, GAD67 and GAD65, which are encoded by *GAD1* and *GAD2*, respectively. To investigate the association between *GAD* genes and temperament in domestic dogs, *Canis familiaris*, we sequenced the full lengths of the coding regions of these genes and identified three single nucleotide polymorphisms (SNPs) in *GAD1* and one in *GAD2*. When comparing genotype and allele frequencies of SNPs among five breeds with different behavioral traits, statistically significant interbreed differences were observed for three SNPs in *GAD1*. These results suggest that *GAD1* SNPs may be useful for behavioral genetic studies in dogs.
KEY WORDS: canine, GAD, SNP.

J. Vet. Med. Sci. 70(10): 1107–1110, 2008

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system. Its function was first noted with regard to its suppressive effect against convulsions caused by excessive administration of glutamate. Almost all GABA is derived from the decarboxylation of glutamate by glutamate decarboxylase (GAD), which exists in two main isoforms, GAD67 and GAD65, named according to their molecular weights (67 and 65 kDa, respectively). GAD67 and GAD65 are each encoded by a single gene, *GAD1* and *GAD2*, and they show overall amino acid homology of about 65%, with a lower degree of similarity in their N-termini, which correspond to exons 1–3 [1, 3]. Both GADs are expressed in the brain and act at the presynaptic terminals [16], but they show differences in their distribution and responsiveness to their common cofactor, pyridoxal 5'-phosphate (PLP). This is thought to result in their distinctive functions: GAD67 has a primary role in maintaining GABA concentrations, and GAD65 is involved in the rapid synthesis of GABA [12].

In *GAD2* knockout mice, changes in conditioned fear behavior were induced in which freezing is reduced and flight and escape behaviors are increased [24]. In addition, the relation between *GAD1* polymorphisms and diseases has been examined based on the effects of benzodiazepines—which stimulate the GABA pathway—on psychiatric diseases. One study showed a statistically significant association between single nucleotide polymorphism (SNP) haplotypes in 5'-UTR and introns of the *GAD1* gene and a decreased risk for neuroticism, major depressive disorder, and generalized anxiety disorder [7].

Domestication of dogs began more than 15,000 years ago, and selective breeding based on the desire to develop dogs with particular physical traits, behavioral characteristics, or unique skills has resulted in today's large variety of breeds, which now exceeds 400 [18]. These breed-specific characteristics are likely to reflect the genetic background, and with regard to temperament, neurotransmitter pathways are thought to play a role in controlling mental status in both dogs and humans [9, 19, 21, 27]. Based on this concept, our laboratory and other groups have reported that genetic polymorphisms in some neurotransmitter-associated genes show interbreed differences, suggesting that these polymorphisms may be associated with breed-specific temperament [4, 5, 8, 10, 14, 15, 17, 26, 29]. Some of these polymorphisms were recently reported to be weakly associated with "distractibility" in detection dogs [13] and activity-impulsivity in police dogs [6]. To study the association of GADs with temperament in dogs, we first sequenced and identified polymorphisms of both the *GAD1* and *GAD2* genes and examined their variations among five breeds (Golden Retriever, Labrador Retriever, Maltese, Miniature Schnauzer, and Shiba) which show differences in behavioral traits [28] and are popular in Japan.

We designed consensus primers from the *GAD1* and *GAD2* genes of other species registered in GenBank [accession numbers *GAD1*: NM_000818 (human), NM_008078 (mouse), NM_213895 (pig), *GAD2*: NM_000817 (human), NM_008077 (mouse), NM_213894 (pig)]. Complementary DNA (cDNA) obtained by reverse-transcription of brain mRNA of a beagle which had already been prepared in the previous studies of our laboratory [4, 14, 15, 17, 26] was amplified by polymerase chain reaction (PCR) with Ex *Taq* polymerase (Takara Bio Inc., Otsu, Japan) and the rapid amplification of cDNA ends (RACE) method with a SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, U.S.A.). The full length of coding regions was sequenced directly by the dye-termination method with an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster

* CORRESPONDENCE TO: TAKEUCHI, Y., Laboratory of Veterinary Ethology, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–8657, Japan.
e-mail: aytake@mail.ecc.u-tokyo.ac.jp

City, CA, U.S.A.). The genes amplified for sequencing in this study contained open reading frames of 1785 bp (*GAD1*) and 1758 bp (*GAD2*). These nucleotide sequences of canine *GAD1* and *GAD2* were shown in GenBank with the accession numbers AB261624 and AB261623, respectively. The identified sequences were identical to those obtained from the canine whole genome shotgun sequence (GenBank; NW876303 chromosome 36 for *GAD1* and NW876290 chromosome 2 for *GAD2* gene), and the exon portions of the predicted sequence for *GAD1* (GenBank; XM_535958) and the reported sequence for *GAD2* (GenBank; DQ060442). The respective homologies at the nucleotide level of human, mouse, rat, and pig homologs were 93.7%, 90.9%, 90.3%, and 94.3% (*GAD1*), and 92.2%, 89.2%, 89.0%, and 91.8% (*GAD2*), respectively. Similar to other decarboxylases, GADs require PLP binding for activation, and their PLP binding sites (four amino acid residues: Asn-Pro-His-Lys) are well conserved in mammals with regard to both structure and location [25]. These amino acid residues were also seen in exon 12 of the identified sequences of both *GAD1* and *GAD2* in this study. To search for polymorphisms of the coding regions of *GAD1* and *GAD2* genes, brain cDNA from ten unrelated Beagles were amplified by PCR in 50-μl reaction mixtures with 15 *n*g of cDNA, primers, and Ex *Taq* polymerase (Takara Bio Inc.) using standard methods as described in Table 1.

By comparing the sequences of the PCR products, we identified two SNPs at nucleotides 339 (A/C), and 1005 (A/G) of the *GAD1* gene, and one SNP at nucleotide 1095 (T/C) of the *GAD2* gene. Addition to these, one SNP at nucleotides 1021(G/A) of the *GAD1* gene was detected in the process of genotype determination described later. Only the G1021A SNP on *GAD1* was non-synonymous, causing an amino acid substitution on the 341st valine to isoleucine, while the other three were silent. The part of amino acid sequence including the G1021A SNP of canine *GAD1* gene was completely identical to those of other mammals (human, mouse, rat, pig, cat, and cow; GenBank: Q99259, NP_032103, NP_058703, P48319, NP_001009225, and Q0VCA1, respectively). This identity also extends to other vertebrates, such as chicken and *Xenopus* (GenBank: NP_990244 and NP_001039075), and even to invertebrates (*Drosophila*, GenBank: NP_523914). This evolutionary conservation has been suggested to be important in candidate gene association studies, and SNPs that result in alterations in conserved amino acids are more likely to contribute to changes in function [20, 30]. The G1021A SNP in *GAD1* is one such case, and may be associated with functional effects. To assess the genetic variation in the four identified polymorphisms, samples of peripheral blood were obtained from 193 dogs of five different breeds (Golden Retriever, Labrador Retriever, Maltese, Miniature Schnauzer and Shiba) as used in the previous studies of our laboratory [4, 14, 15, 17, 26]. Genomic DNA was extracted and amplified by PCR in 50-μl reaction mixtures with 15–20 *n*g of genomic DNA, primers, and Ex *Taq* polymerase (Takara Bio Inc.) using standard methods as described in Table 1. The amplified fragments were sequenced directly for genotyping of polymorphisms, and genotype and allele frequencies were compared among breeds using the χ^2 test.

The genotype and allele frequencies of the *GAD1* polymorphisms in each of the five breeds are presented in Table 2. The A339C polymorphism was seen in all five breeds with statistically significant interbreed differences in the actual number of genotypes (χ^2=96.193, df=8, P<0.0001) and alleles (χ^2=144.52, df=4, P<0.0001). The A allele was dominant in the Golden Retriever, Labrador Retriever, and Maltese, whereas the C allele was dominant in the other two breeds. The A1005G polymorphism was not seen in the Golden Retriever or the Shiba, and was rarely seen in the Labrador Retriever and the Maltese. The interbreed differences in the genotypes and alleles between Miniature Schnauzer, Labrador Retriever, and Maltese were statistically significant (χ^2=47.247, df=4, P<0.0001 and χ^2=62.271, df=2, P<0.0001, respectively). The G1021A polymorphism was not seen in the Golden Retriever, Labrador Retriever, or the Miniature Schnauzer, was rarely seen in the Maltese, and the interbreed differences in the genotypes and alleles between the Shiba and Maltese were statistically significant (χ^2=18.119, df=2, P=0.0001 and χ^2=16.374, df=1, P<0.0001, respectively). With regard to the T1095C polymorphisms of *GAD2*, all of the dogs from the five breeds examined had the TT genotype. When we first searched for the existence of polymorphisms in ten bea-

Table 1. Primers, annealing temperature, Ex *Taq* polymerase and PCR product size for polymorphism identification and genotyping

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Annealing temperature (°C)	Ex *Taq* Polymerase (U)	PCR product (bp)
Identification of polymorphism					
GAD1 5' end	agctgatggcgtcttcgac	ggtatcctgcacacatctgg	62	1.00	1308
GAD1 3' end	aagtatggggtccgtacagg	gtcctagagtgccggaagga	60	1.25	1315
GAD2 5' end	cgccacactcctacacacac	tcacgctgtctgttccaatc	62	1.00	1000
GAD2 3' end	tgatcgcacgctttaagatg	aggggcaatttggcactatt	58	1.25	1345
Genotyping					
GAD1-A339C	cctggaacaaatgaggtctga	ggtggagcgatcaaatgtct	58	1.00	215
GAD1-A1005G and G1021A	gctgtattcccttgcagctc	acatcgacatgcagccatag	58	1.25	351
GAD2-T1095C	tgcttaatgggtccttcacc	gccaaccacagactccagtt	57	1.25	709

Table 2. Genotype, allele frequencies and heterozygosities of polymorphisms on *GAD1* gene in five dog breeds

Breed		Genotype			Allele		H-obs
A339C	n[a)]	AA	AC	CC	A	C	
GLD	47	17 (36.2)	17 (36.2)	13 (27.6)	51 (54.3)	43 (45.7)	0.362
LAB	41	26 (63.4)	14 (34.2)	1 (2.4)	66 (80.5)	16 (19.5)	0.342
MLT	40	13 (32.5)	18 (45.0)	9 (22.5)	44 (55.0)	36 (45.0)	0.450
MS	26	0 (0.0)	6 (23.1)	20 (76.9)	6 (11.5)	46 (88.5)	0.231
SHIBA	38	0 (0.0)	4 (10.5)	34 (89.5)	4 (5.3)	72 (94.7)	0.105
Total	192	56 (29.2)	59 (30.7)	77 (40.1)	171 (44.5)	213 (55.5)	0.307
A1005G	n	AA	AG	GG	A	G	
GLD	47	47 (100.0)	0 (0.0)	0 (0.0)	94 (100.0)	0 (0.0)	–
LAB	41	39 (95.1)	2 (4.9)	0 (0.0)	80 (97.6)	2 (2.4)	0.049
MLT	40	38 (95.0)	2 (5.0)	0 (0.0)	78 (97.5)	2 (2.5)	0.050
MS	26	9 (34.6)	11 (42.3)	6 (23.1)	29 (55.8)	23 (44.2)	0.423
SHIBA	39	39 (100.0)	0 (0.0)	0 (0.0)	78 (100.0)	0 (0.0)	–
Total	193	172 (89.1)	15 (7.8)	6 (3.1)	359 (93.0)	27 (7.0)	0.078
G1021A	n	GG	GA	AA	G	A	
GLD	47	47 (100.0)	0 (0.0)	0 (0.0)	94 (100.0)	0 (0.0)	–
LAB	41	41 (100.0)	0 (0.0)	0 (0.0)	82 (100.0)	0 (0.0)	–
MLT	40	39 (97.5)	0 (0.0)	1 (2.5)	78 (97.5)	2 (2.5)	–
MS	26	26 (100.0)	0 (0.0)	0 (0.0)	52 (100.0)	0 (0.0)	–
SHIBA	39	23 (59.0)	13 (33.3)	3 (7.7)	59 (75.6)	19 (24.4)	0.333
Total	193	176 (91.2)	13 (6.7)	4 (2.1)	365 (94.6)	21 (5.4)	0.067

The percentage in each category is shown in parenthesis.
H-obs; observed heterozygosity
GLD; Golden Retriever, LAB; Labrador Retriever, MLT; Maltese, MS; Miniature Schnauzer.
a) Reduction of total number from 193 to 192 (*GAD1*-A339C) was due to a failure in PCR amplification.

gles, nine dogs had TT and the other had TC. Thus, the T1095C polymorphism in *GAD2* appears to be rare and/or characteristic of beagles. For each breed, there were no deviations from Hardy-Weinberg equilibrium in the distributions of A339C, A1005G, or G1021A on *GAD1*. According to the SNP databases generated by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/snp_blastByOrg.cgi) and the Broad Institute (http://www.broad.mit.edu/ftp/pub/papers/dog_genome/snps_canfam2/snp_lists/), there were no previously reported SNPs within exon regions of *GAD1* gene, therefore all the three SNPs identified in this study were shown to be novel.

The domestic dog is regarded as a good model for studies on behavioral genetics, as a variety of breeds have been developed by selective breeding for their physical traits and behavioral characteristics [9, 23, 27]. This has resulted in differences in gene expression from the wild wolf ancestor, especially in the hypothalamus, which controls specific emotional, endocrinological, and autonomic responses of dogs and is highly conserved in all mammals [22]. Based on these features in dogs, several genes related to neurotransmitter pathways have been examined as candidates associated with temperament, and 22 exonic polymorphisms on 10 genes have been reported to show interbreed differences in their genotype and allele frequencies [4, 10, 14, 15, 17, 26, 29]. However, studies on dog temperament are not progressing well due mainly to the lack of reliable means to assess temperament, which are essential for genetic association investigations [2, 11]. Given reliable assessments of temperament applicable to variable breeds, the increasing information on the dog genome supported by the recent publication of the full-sequence of a female boxer by the Broad Institute could be used in such studies. Likewise, the genetic polymorphisms associated with breed differences, including SNPs in the *GAD1* gene reported here, are expected to aid in identifying temperament-associated genes in dogs. This will contribute to human-dog relationships, such as the prediction of compatibility of pet dogs with their owners and suitability of working dogs.

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