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*Research Article*

# High Frequency of a Single Nucleotide Substitution (c.-6-180T>G) of the Canine *MDR1/ABCB1* Gene Associated with Phenobarbital-Resistant Idiopathic Epilepsy in Border Collie Dogs

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A single nucleotide substitution (c.-6-180T>G) associated with resistance to phenobarbital therapy has been found in the canine *MDR1/ABCB1* gene in Border Collies with idiopathic epilepsy. In the present study, a PCR-restriction fragment length polymorphism assay was developed for genotyping this mutation, and a genotyping survey was carried out in a population of 472 Border Collies in Japan to determine the current allele frequency. The survey demonstrated the frequencies of the T/T wild type, T/G heterozygote, and G/G mutant homozygote to be 60.0%, 30.3%, and 9.8%, respectively, indicating that the frequency of the mutant G allele is extremely high (24.9%) in Border Collies. The results suggest that this high mutation frequency of the mutation is likely to cause a high prevalence of phenobarbital-resistant epilepsy in Border Collies.

## 1. Introduction

Recently, a single nucleotide substitution associated with phenobarbital-resistant idiopathic epilepsy was found in Border Collies [1], which frequently present with severe epileptic seizures that are poorly controlled with antiepileptic drugs [2]. This polymorphic mutation is a substitution of thymine for guanine at intron 1 near the 5′-end of the canine *MDR1*/*ABCB1* gene (c.-6-180T>G), where the most important promoter elements are located [3]. The mutation is not directly associated with the pathogenesis of idiopathic epilepsy, but it may create resistance to phenobarbital therapy in epileptic Border Collies [1].

However, the c.-6-180T>G mutation has been shown not to be a fully penetrant polymorphic mutation for phenobarbital nonresponsiveness because phenobarbital-resistant, idiopathically epileptic Border Collies in a previous case control study included a homozygous T/T wild-type dog [1]. On the basis of data from a relatively small cohort of Border Collies, it was hypothesized that the mutation might be related to an upregulation of the gene and an overexpression of P-glycoprotein (P-gp) encoded by the *MDR1*/*ABCB1* gene. Therefore, further studies are required to understand the regulatory effect of the mutation and its potential clinical relevance. For these purposes, an accurate method of genotyping for the mutation should be developed, and the frequency of the mutation should be determined in a large, normal population of dogs.

In the present study, a PCR-restriction fragment length polymorphism (RFLP) assay was developed in order to discriminate the genotypes of the mutation, and a genotyping survey was conducted in Japan by using samples from clinically healthy Border Collies in order to determine the current frequency of the mutant allele.

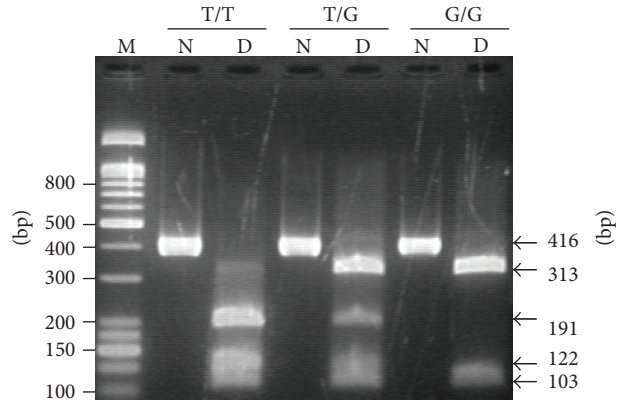


Figure 1: Electrophoretogram of agarose gel using PCR-restriction fragment length polymorphism assay. Fragment patterns in the 3 genotypes such as homozygous wild type (T/T), heterozygote (T/G), and homozygous mutant type (G/G) are shown with molecular size markers (M). The amplified DNA without digestion (N) and DNA digested with restriction endonuclease *Mbo*I (D) were analyzed simultaneously by electrophoresis. bp: base pairs.

## 2. Methods and Materials

Control samples of each genotype, which were determined by direct DNA sequencing, were used to evaluate the accuracy of the genotyping assay. These samples were collected from 4 wild-type (T/T), 9 heterozygous (T/G), and 10 homozygous mutant-type (G/G) dogs. DNA templates were prepared using saliva spotted onto Flinders Technology Associates filter paper (FTA card, Whatman International Ltd., Piscataway, NJ, USA). For the PCR-RFLP assay, a 1.2 mm disc punched out of the FTA card was used as a template after quick washing as reported previously [4]. The PCR test was performed by targeting the sequence around the mutation with forward (5′-GCA GTG GGG TGA GAA CTA GA-3′) and reverse (5′-CGC AAG CCA TGT AAG GTA TG-3′) primers in a 20 $\mu$L reaction mixture containing 10 $\mu$L of a 2× PCR master mix (GoTaq Hot Start Green Master Mix, Promega Corp., Madison, WI, USA), 12.5 pmol of primers, 1 $\mu$L of GC enhancer solution (360 GC Enhancer, Applied Biosystems, Foster City, CA, USA), and the treated disc of the FTA card as a template. After denaturation at 95°C for 10 min, 45 cycles of amplification were carried out at a denaturing temperature of 95°C for 30 sec, an annealing temperature of 60°C for 30 sec, and an extension temperature of 72°C for 30 sec. Extension during the last cycle was performed at 72°C for 7 min and 30 sec. The PCR product was digested with a *Mbo*I restriction endonuclease (New England Biolabs Inc., Ipswich, MA, USA) at 37°C for 90 min in a 10 $\mu$L reaction mixture containing 8 $\mu$L of the PCR product, 5 U of *Mbo*I, and 1 $\mu$L of 10× restriction enzyme buffer (10× NE buffer, New England Biolabs Inc.) included by the manufacturer. Both the unprocessed and digested PCR products were subjected to electrophoresis in 3% agarose gel (Agarose 21, Nippon Gene Co., Ltd., Tokyo, Japan). The PCR-RFLP assay was designed to digest the wild-type sequence [↓GATC] into 3 fragments and to not digest the mutant sequence [GAGC] resulting in 2 fragments.

The genotyping survey was carried out by using DNA templates extracted from saliva samples of 472 Border Collies aged 2 months to 14 years in Japan. These samples were collected between 2006 and 2013 by the Japan Border Collie Health Network, a volunteer breeders' association with the owners' informed consent. The PCR-RFLP assay established in this study was used for genotyping.

## 3. Results

As shown in Figure 1, a 416-base pair (bp) DNA band was amplified, in theory, in all the genotypes. In the G/G homozygous mutant-type dog, the amplified band was digested into 2 fragments (i.e., 313 and 103 bp bands) because there was only 1 restriction site available to the *Mbo*I, which was present in all the genotypes and unrelated to the target sequence. In the T/T homozygous wild-type dog, the amplified band was digested into 3 fragments (i.e., 191, 122, and 103 bp bands) because there were 2 restriction sites available: one at the target sequence [↓GATC] and another at the same sequence present in all the genotypes. The amplification product in the T/G heterozygous dog was digested into 4 fragments (i.e., 313, 191, 122, and 103 bp bands). Owing to the inseparability of the 122 and 103 bp bands, these 2 bands externally appeared to be 1 band; therefore, the digested PCR products in the T/T wild-type and T/G heterozygous dogs externally appeared as 2 and 3 fragments, respectively. The genotypes of all the dogs examined were consistent with the results of direct DNA sequencing.

In the survey on 472 Border Collies, 283 (60.0%) were T/T wild-type dogs, 143 (30.3%) were T/G heterozygous dogs, and 46 (9.8%) were G/G homozygous mutant-type dogs. The overall frequency of the mutant G allele was 24.9%.

## 4. Discussion

Pharmacogenetics is a relatively new discipline that investigates how genetic variations are related to drug response, and it is expected to be an important tool for developing personalized medicine [5]. Pharmacogenetic biomarkers relevant to various diseases, drugs, and genes have been found and clinically used to maximize therapeutic efficacy, reduce adverse drug reactions, and determine the most appropriate drug dosage required for efficacious and safe treatment [6]. Information about pharmacogenetics in domestic animals is still not as extensive as in humans; however, in veterinary medicine, there is great potential for advances in the coming years because whole-genome sequencing of many species has been completed [7]. The genetic marker, c.-6-180T>G, associated with phenobarbital-resistant idiopathic epilepsy in dogs may be an important pharmacogenetic biomarker in veterinary medicine in the near future.

In the PCR-RFLP assay developed in the present study, all the genotypic variations of c.-6-180T>G could be easily discriminated by confirming the presence and/or absence of 313 and 191 bp fragments via agarose gel electrophoresis

(Figure 1). The restriction site that is present in both alleles and unrelated to the target sequence did not interfere with the determination of all the genotypes but provided assurance of the digestive function of the restriction endonuclease. Furthermore, other genotyping assays (including a TaqMan probe-based real-time PCR method) could not be established because nucleic acid sequences around the mutation are rich in GC pairs and include a partially duplicated repeat (GenBank reference no. NC_006596 and accession no. AAEX03009295.1), which might interfere with specific hybridization by designed probes. Therefore, the PCR-RFLP assay is a very useful tool for the genotyping of c.-6-180T>G.

The genotyping survey showed that the mutant G allele frequency in Border Collies is extremely high (approximately 25%) compared to that in other molecularly defined, fatal inherited diseases in Border Collies, such as neuronal ceroid lipofuscinosis (4.1%) [8] and trapped neutrophil syndrome (5.6%) [9]. The high frequency of the G allele in Japanese Border Collies cannot be generalized to Border Collie populations in different countries, however, because population stratification can easily occur in a geographic region unknowingly, by using dogs with a single genotype for breeding in pure dog breeds (i.e., the founder effect) [10]. The G allele frequency in this study was consistent with that in a small population of European Border Collies (24%), although this population did not consist of randomly selected, normal animals [1].

The phenotype of the mutant G allele seems to act in a dominant fashion [1]: as a consequence, approximately 40% Border Collies (G/G and T/G genotypes) examined in this study may show resistance to phenobarbital therapy if they become affected with epilepsy. This might be the reason why drug-resistant epilepsy frequently occurs in Border Collies, although there are other possible causes [2, 11]; further studies are needed to clarify this issue.

Previous studies hypothesized that phenobarbital resistance is due to an upregulation of the *MDR1*/*ABCB1* gene and the subsequent overexpression of this gene's product, P-gp, in the brains of Border Collies with drug-resistant epilepsy [1]. P-gp is expressed at the luminal membrane of the endothelial cells of brain capillaries and restricts the entry of drugs (including ivermectin and phenobarbital) into the central nervous system by an efflux-based transport mechanism [4, 12]. P-gp is also expressed in various mammalian tissues such as the brush border membrane of epithelial cells in the intestinal tract, the luminal membrane of proximal tubules in the kidney, and the canalicular membrane of liver hepatocytes [13]. Overexpression of P-gp in these organs diminishes oral drug bioavailability and promotes drug elimination into urine and bile [14]. Therefore, c.-6-180T>G in Border Collies may not only affect phenobarbital resistance but may also affect the pharmacokinetics of other P-gp substrate drugs. Additionally, in veterinary medicine, there is another well-known mutation in the canine *MDR1*/*ABCB1* gene (c.227_230del) that causes a P-gp defect and, subsequently, substrates drug intoxication, including ivermectin toxicosis [15]; however, the allele frequency of c.227_230del is low (0.25%) in Border Collies [4]. It is necessary to further study the association between P-gp and c.-6-180T>G and the interaction between these mutations in the canine *MDR1*/*ABCB1* gene before the pharmacogenetic information related to c.-6-180T>G can be applied to clinical practice.

## Conflict of Interests

The authors have no conflict of interests to declare.

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