Detection of the ATP Binding Cassette Subfamily B Member 1 Gene Mutation in Dogs

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ABSTRACT

To investigate the base deletion and frequency of the canine ATP binding cassette subfamily B member 1 (ABCB1) gene in Nanjing, China, genomic DNA was extracted from the blood or oral swab samples of 246 dogs from the Veterinary Teaching Hospital of Nanjing Agricultural University. The ABCB1 gene fragment was amplified by PCR. The PCR products were subjected to 12% neutral polyacrylamide gel electrophoresis, followed by silver nitrate staining to compare the differences in the bands. The bands were cut and sequenced. The results showed that five Scottish shepherds and one Shetland sheepdog had 4-bp base deletions in ABCB1, and all were heterozygous deletions, missing the ATAG sequence. Moreover, the Shetland sheepdog showed point mutations. These results suggest that the ABCB1 gene base deletion occurred in Scottish shepherds and Shetland sheepdogs in the Nanjing area, and the base deletion frequencies were 38.5% and 14.3%, respectively. These results should help provide a risk estimation for dogs who are administered ABCB1-related drugs in clinical therapy.

INTRODUCTION

P-glycoprotein is an important transporter protein encoded by ATP binding cassette subfamily B member 1 (ABCB1) also named the multidrug resistance gene (MDR1), which significantly affects drug metabolism.[1] P-glycoprotein can pump noxious substrates from cells into the blood to protect cells from poisoning. P-glycoprotein is expressed in epithelial cells of the renal tubules, liver, and intestines, and in the capillary endothelium of the blood-testis and blood-brain barriers. [2]

Dogs with a 4-bp deletion in the ABCB1 gene show intolerance to certain drugs routinely used in veterinary medicine, such as ivermectin.[3,4] This mutation leads to a dysfunctional P-glycoprotein drug transporter, which results in drug accumulation in

the brain and severe neurotoxicity. ABCB1 gene mutations have been reported in many countries, including the United States, Japan, Germany, Australia, France, and the United Kingdom.[5] The canine ABCB1 gene mutation is mainly a 4-bp deletion and a single nucleotide substitution as reported in collies and many other dog breeds in many countries.[6] The 4-bp deletion results in a premature stop codon and a truncated, non-functional protein product. Drugs are then accumulated in the brain, leading to severe neurotoxicity.[7] Currently, few reports exist on the ABCB1 gene mutation in China,[8] and no ABCB1 gene mutations in dogs in Nanjing have been reported. It is unclear which species in this region have ABCB1 gene mutations. Therefore, we examined the mutations in exon 4 of ABCB1 gene in dogs in Nanjing, to provide guidance for clinical medication and breeding.

MATERIALS AND METHODS

Sample Collection

All samples were collected from dogs presented at the Veterinary Teaching Hospital of Nanjing Agricultural University. In total, 246 samples were collected, including 220 blood samples and 26 oral swabs. One hundred and eighty samples were collected from clinically healthy, client-owned dogs. The other samples were collected from patients which suffered from diseases such as fracture, contusion, cough, skin disease, diarrhea, urinary stone, and so on. There was no clinically symptom associated with neurotoxicity caused by drugs.

The age of these dogs ranged from 2 months to 14 years. These dogs included Scottish shepherds (n=13), Shetland sheepdogs (n=7), Pembroke Wales corgis (n=12), border shepherds (n=9), Siberian Eskimo dogs (n=10), poodles (n=60), golden retrievers (n=10), a Yorkshire terrier (n=1), Labrador retrievers (n=9), Alaskan sled dogs (n=4), Pomeranians (n=7), bulldogs (n=3), Chow Chows (n=1), Bichon Frises (n=10), Chihuahuas (n=2), Cocker spaniels (n=3), Pekingese (n=3), Shih Tzus (n=2), pit bulls (n=1), Dachshunds (n=3), Maltese dogs (n=1), Samoyeds (n=8), Beagles (n=2), German shepherds (n=5), Schnauzers (n=4), pugs (n=7), butterfly dog (n=1), Whitby dog (n=1), Springer spaniels (n=3), miniature pinschers (n=2), bull terriers (n=1), Germany Weimar dog (n=1), Akita (n=1), and mixed breed dogs (n=39).

Blood samples were placed into EDTA tubes and stored at -20 ºC until further use. Epithelial cell samples from the oral cavity were obtained by swiping the inside of a cheek with a cotton swab. The cotton swabs were dipped into 1-mL physical saline tubes and stored at -20 ºC until further use. The samples were numbered as follows: A1-A23, B1-B14, C1-C14, D1-D24, E1-E24, F1-F24, G1-G23, H1-H14, I1-I18, J1-J24, and K1-K24 and L1-L20. Samples were collected in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Jiangsu province. The protocol was approved by the Committee on the Ethics of Animal Experiments of Jiangsu province.

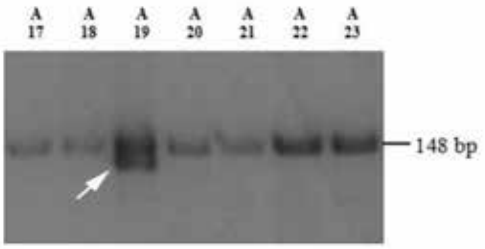
Genomic DNA Extraction

Canine genomic DNA was extracted by protease K digestion and phenol extraction as described by Sambrook et al.[9] with minor modifications. Briefly, 100 µL of whole blood or oral swab samples were mixed with 400 µL of cell lysis buffer (10 mmol/L Tris-HCl pH 8.0, 0.1 mol/L EDTA, 0.5% SDS) and incubated at 37 ºC for 1 h. The mixture was then digested by protease K (100 µg/mL) for 2 h at 50 ºC. The DNA was extracted with phenol, precipitated by ethanol and dissolved in 50 µL of TE buffer (10 mmol/L Tris-HCl, 1 mol/L EDTA, pH 8.0).

Primer Design and Synthesis

PCR primers were designed per the canine ABCB1 gene sequence (accession number AF045016), which can amplify 148-bp wild-type fragments and 144-bp mutant fragments located in the 4 exon of the ABCB1 gene per Mealey's report.[6] Primer sequences were as follows: MDR1 F: 5'-GGCTTGATA GGTTGTATATGTTGGTG-3' and MDR1 R: 5'-ATTATAACTGGAAAAGTTTTGTT T-3'. Primers were synthesized by Invitro-

Figure 1. *ABCB1 gene mutation detection by 12% neutral polyacrylamide gel electrophoresis (partial sample results). Two bands appeared, one was a 148-bp wild-type fragment, and the other was 144-bp mutant fragment (arrow).*



gen (Shanghai, China).

PCR Amplification of the ABCB1 Gene

The PCR reaction was as follows: 2×Taq PCR Mix; 12.5 μL (TIANGEN, Beijing, China); MDR1 F (10 μmol/L), 0.5 μL; MDR1 R (10 μmol/L), 0.5 μL; and DNA template, 5 μL, replenished to 25 μL with ddH2O. The PCR amplification conditions were as follows: pre-denaturation at 95 ºC for 5 min, followed by 35 cycles of denaturation at 95 ºC for 15 s, annealing at 52 ºC for 15 s, extension at 72 ºC for 30 s, and extension at 72 ºC for 7 min.

Neutral Polyacrylamide Gel Electrophoresis Analysis

Neutral polyacrylamide gel (12%) was made as follows: 20.0 mL of acrylamide:methylene bisacrylamide (29:1), 10.0 mL of 5×TBE (54 g/L of Tris, 27.5 g/L of boric acid, 20 mL of 0.5 mol/L EDTA, pH 8.0), 0.35 mL of 10% (m/V) ammonium persulfate, 35 μL of TEMED and 19.65 mL of H2O. PCR products (25 μL) were diluted with 55 μL of 1 × loading buffer, and the 7-μL samples were added into the gel wells. The gels were run at 120 V for approximately 12 h with 1×TBE running buffer on a DCode universal mutation detection system (Bio-Rad, USA). After electrophoresis, the gels were silver stained using the method described by Sanguinetti et al.[10]

Purification and Sequencing of PCR Products

The small piece of gel containing the desired DNA fragment was excised carefully with a scalpel, washed three times with sterile water, and transferred into a new tube with 100 μL of TE buffer for 24 h. The DNA solution was used as a template for ABCB1 gene PCR amplification as previous description. After purification of PCR products (TIANgel Midi Purification Kit, TIANGEN, Beijing, China), bi-directional DNA sequencing with PCR primers (MDR1F and MDR1R) on an ABI-Prism 3100 Genetic Analyzer (Applied Biosystems, USA) were performed. The sequencing results were analysis using DNAstar software. Sequence alignment was performed using Blastn, which is available on the NCBI server (http://www.ncbi.nlm.nih.gov).

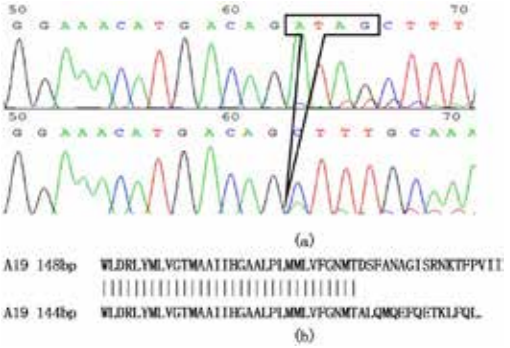
RESULTS

DNA was extracted from the 246 samples, and the partial gene fragment located in the 4 exon of ABCB1 gene was amplified by PCR. The results are shown in Figure 1. The 148-bp fragment was amplified from all samples; however, a 144-bp fragment was also amplified from six dog samples (A19, D4, H14, J2, K4 and L15), in addition to the 148-bp fragment. These results suggested that those dogs carried an ABCB1 heterozygous mutation. The mutation ratio in all dogs was 2.4%.

Samples A19, D4, H14, J2 and L15 were collected from Scottish shepherds, and sample K4 was collected from a Shetland Sheepdog. Therefore, five dogs had heterozygous mutations in the 13 Scottish shepherd samples, with a mutation rate of 38.5% (carriers), and no homozygous mutations were found. Only one dog (sample K4) carried a heterozygous mutation in the 7 Shetland Sheepdog samples, with a mutation rate of 14.3%.

The 148-bp and 144-bp DNA amplification fragments from the silver-stained polyacrylamide gels were recovered and amplified again by PCR using primers MDR1 F and MDR1 R. Sequencing results showed that the size of the ABCB1 genes from G4 and G13 were only the 148-bp

Figure 2. *DNA and protein sequence alignment between the 148-bp and 144-bp fragments amplified from A19 (a Scottish shepherd). (a) A 4-bp deletion (b) Due to a 4-bp deletion, the protein sequence was altered after the deletion position.*



indicated wild-type. However, the size of the ABCB1 genes of A19, D4, H14, J2, L15 and K4 were 148-bp, and 144-bp indicated heterozygous mutations. Four base differences ("ATAG") were noted between the 148 bp and 144 bp in A19 (the Scottish shepherd, Fig 2a-b) and K4 (the Shetland sheepdog, Fig 3a-b). The 4-bp deletion mutation was also found in the other four Scottish shepherds (D4, J2, L15, and H14). A 4-bp deletion altered the amino acid sequence after the deletion position (Fig 2b and Fig 3b). A premature stop codon also occurred before the end of the sequence, resulting in a truncated, nonfunctional protein product.

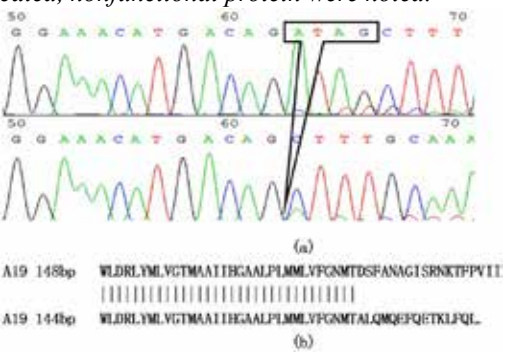
Sequence analysis of the no. K4 Shetland sheepdog is shown in Figure 3. It also had a heterozygous mutation with the 4-bp deletion of "ATAG" and showed base mutations, changing "AATG" to "CAAT" (Figure 3a). In addition, an amino acid mutation beside the amino acid sequence alteration and a truncated, nonfunctional protein were noted (Figure 3b).

DISCUSSION

To evaluate the breed distribution of this mutation in Australia in 2005, the ABCB1 gene from 33 collies, 17 Australian shepherds, 7 border shepherds and 7 Shetland Sheepdogs were detected.[6] PCR results showed that the mutant ABCB1 gene was a 4-bp deletion. Four collies (12%) carried homozygous normal alleles (normal), 21 collies (64%) were heterozygous (carriers), and 8 collies (24%) were homozygous for the mutant allele (affected). The rates in Australian shepherds were 36% normal, 43% carriers, and 21% affected. Shetland sheepdogs were 37% heterozygous, with no border shepherd mutations in this research. To evaluate the breed distribution of this mutation in Germany in 2010, 7,378 dogs were screened, including 6,999 purebred and 379 mixed breed dogs.[5] The 4-bp deletion was present in collies, longhaired whippets, Shetland sheepdogs, miniature Australian shepherds, Australian shepherd, Wällers, white Swiss shepherds, Old English sheepdogs, and Australian shepherds.

In this study, the canine ABCB1 gene of 246 dogs in Nanjing was detected by PCR and sequencing. The results showed mutations in five Scottish shepherds and a Shetland sheepdog, and all were heterozygous. The ABCB1 gene showed a 4-bp base deletion, and the four missing bases were "ATAG," which was the same as other reported deletions in this gene.[4] In this study, 61.5% (8/13) of the Scottish shepherds studied were homozygous for the normal allele (normal), 38.5% (5/13) were heterozygous (carriers), and none were homozygous

Figure 3. *DNA and protein sequence alignment between the 148-bp and 144-bp fragment amplified from the no. K4 Shetland sheepdog. (a) A 4-bp deletion and base mutations, (AATG to CAAT). (b) An amino acid mutation beside the altered amino acid sequence and a truncated, nonfunctional protein were noted.*



(affected). For Shetland sheepdogs, the data showed 85.7% were normal, 14.3% were carriers and none were affected. No mutations were found in other dogs.

In a 2013 study, a single nucleotide substitution (c.-6-180T>G) associated with resistance to phenobarbital therapy was found in the canine ABCB1 gene in border collies.[11] The frequency of single nucleotide substitutions was quite high in Japanese border collies, at approximately 24.9%. The mutation was hypothesized to be related to an upregulation of the gene and an overexpression of the P-glycoprotein encoded by the ABCB1 gene. P-glycoprotein overexpression in some organs diminishes oral drug bioavailability and promotes drug elimination into the urine and bile.[2] Therefore, a single nucleotide substitution in border collies may not only affect phenobarbital resistance, but may also affect the pharmacokinetics of other P-glycoprotein substrate drugs.[11]

In addition to the above 4-bp deletion in this research, point mutations were found at 3 loci in the Shetland sheepdog, changing "AATG " to "CAAT." The point mutations in the Shetland sheepdog found here were previously unreported. Unfortunately, only one case was found. Therefore, we are uncertain whether the mutations affected ABCB1 gene function.

In conclusion, a 4-bp deletion in the ABCB1 gene was found in dogs in the Nanjing area. Most were Scottish shepherds and 38.5% of these Scottish shepherds were heterozygous ABCB1 gene carriers. Only one Shetland sheepdog presented point mutations in addition to the 4-bp deletion in the ABCB1 gene. These results should help provide a risk estimation for dogs administered ABCB1-related drugs in clinical therapy.

CONFLICT OF INTEREST

The authors confirm that they do not have any conflict of interest.

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