Establishment of a PCR analysis method for canine BRCA2

Yasunaga Yoshikawa1*, Masami Morimatsu2, Kazuhiko Ochiai3, Kento Okuda1, Takahiro Taoda4, Seishiro Chikazawa5, Asako Shimamura6, Toshinori Omi3, Makoto Bonkobara6, Koichi Orino1 and Kiyotaka Watanabe1

Abstract

Background: Mammary tumors are the most common tumor type in both human and canine females. In women, carriers of mutations in BRCA2, a tumor suppressor gene product, have a higher risk of breast cancer. Canine BRCA2 has also been suggested to have a relationship with mammary tumors. However, clearly deleterious BRCA2 mutations have not been identified in any canine mammary tumors, as appropriate methods to detect mutations or a consensus BRCA2 sequence have not been reported.

Findings: For amplification and sequencing of BRCA2, we designed 14 and 20 PCR primer sets corresponding to the BRCA2 open reading frame (ORF) and all 27 exons, respectively, including exon-intron boundaries of the canine BRCA2 regions, respectively. To define the consensus canine BRCA2 ORF sequence, we used established methods to sequence the full-length canine BRCA2 ORF sequence from two ovaries and a testis obtained from individual healthy mongrel dogs and partially sequence BRCA2 genomic sequences in 20-56 tumor-free dogs, each aged over 6 years. Subsequently, we compared these sequences and seven previously reported sequences, and defined the most common base sequences as the consensus canine BRCA2 ORF sequence. Moreover, we established a detection method for identifying splicing variants. Unexpectedly, we also identified novel splicing variants in normal testes during establishment of these methods.

Conclusions: The present analysis methods for determining the BRCA2 base sequence and for detecting BRCA2 splicing variants and the BRCA2 ORF consensus sequence are useful for better understanding the relationship between canine BRCA2 mutation status and cancer risk.
reported the mutation analysis of full-length of canine BRCA2, but they used many primer sets (about 50 sets) and amplified sequence only from genomic DNA [15]. To establish a more efficient mutation analysis method for cDNA and genomic DNA that requires fewer primer sets, we designed 14 and 20 primer sets in order to sequence the BRCA2 ORF and all 27 exons, respectively, including the exon-intron boundaries of the canine BRCA2 regions. All PCR targets were successfully amplified, and were sufficient to determine DNA base sequences (Figure 1A and 1B).

Some splicing variants of tumor suppressor genes (e.g., BRCA2) in tumor tissue have been associated with tumorigenesis because these splicing variants often lead to frameshift mutations [16,17]. Thus, we next designed five primer sets for detecting splicing variants from cDNA (Figure 1C). All PCR targets were successfully amplified, and the predicted sizes of PCR products were confirmed. During the establishment of this method, we unexpectedly identified splicing variants between exon 10 and exon 14 in normal testes (Figure 1C and 2). These transcripts skipped most of exon 11, leading to frameshift mutations (Figure 2).

To define the consensus canine BRCA2 ORF, we sequenced the full-length canine BRCA2 ORF in two ovaries and a testis obtained from individual healthy mongrel dogs using the method described here. We identified six single nucleotide variations (516 T > C, 1366 T > G, 2428 T > G, 2609A > C, 4481A > C and 8257 T > C) and two insertion/deletion polymorphisms (7126ins/delGTT and 10204ins/delAAA) (Accession numbers: AB622997, AB622998 and AB622999). None of these variations

![Figure 1](image1.png)

**Figure 1** PCR products amplified by each primer set. (A) cDNA samples prepared from total RNA of each mammary gland were amplified. (B) Genomic DNA from each mammary gland was amplified. (C) Splicing variants of the cDNA from total RNA of the mammary gland and testis were amplified. Primer sets for each lane are shown in Table 1. The “M” indicates the molecular size marker (1-kbp DNA ladder; New England Biolabs). Arrowhead and “**” indicate novel BRCA2 transcript and non-specific PCR products, respectively.
resulted in nonsense or frameshift mutations. To determine the most common base sequences and generate a consensus canine BRCA2 ORF sequence, we compared these three new sequences (six alleles) and the seven previously reported sequences (Accession numbers: AB043895.5, NC_006607.2, Z75664 and FJ464397-FJ464400) (Table 2). The four variations (516 T (103I), 2428 T (740 G), 4481A (1425 T), and 8257 T (2683I)) could be defined as consensus base sequences, but the other four variations (1366 T > G, 2609A > C (K801Q), 7126ins/delGTT, and 10204ins/delAAA) could not be defined as such because the frequencies between the major and minor alleles in each variation were nearly identical. We therefore sequenced these four variations in genomic DNA from 20-23 normal blood samples from tumor-free dogs aged over 6 years; the methods described here were used (Tables 3 and 4). We finally defined the most common base sequences as the consensus canine BRCA2 sequence (Table 2). The 10204insAAA variation was consensus sequence in dogs, but in four miniature Dachshunds this variation was determined to be a minor variation (allele ratio; del:ins = 6:2, Table 4).

Figure 2 Identification of splicing variants within exon 11 in normal testes. The splicing variants identified in normal testes lacked a large portion of exon 11. (A) To confirm the presence of a novel BRCA2 transcript, splicing variants of the cDNA from total RNA of the testes were amplified using nested PCR. (B-E) Electropherogram showing the direct sequencing data and overview of the novel BRCA2 transcript that lacked nucleotides 2390 to 6429 (B), 2390 to 6429 and 6588 to 6908 (C), 2390 to 3380, and 4649 to 6429 (D) and 2085 to 2321 and 2390 to 6429 (E) from Testis-1, -3, -4, and -5, respectively. The Testis-1 was the same sample used to generate the data in Figure 1C. Primer sets for each lane are shown in Table 1. The “M” indicates the molecular size marker (1-kbp DNA ladder). Arrowheads indicate novel BRCA2 transcripts.

Yoshikawa et al. BMC Research Notes 2012, 5:173
http://www.biomedcentral.com/1756-0500/5/173
Page 3 of 10
| Primer sets | Forward | Reverse | Annealing temperature | Elongation time | Lane Number | Expected sizes |
|-------------|---------|---------|-----------------------|----------------|-------------|----------------|
| For amplification of cDNA | 1 | 5'-GCCGCAACTCGGAAAGGC-3' | 5'-CCCCAAAACCTTGACTTTTAGC-3' | 60°C | 1 min | Figure 1 A 1 | 834 bp |
| | 2 | 5'-GATCGGTTTATCCCTGTGGTC-3' | 5'-CTTCAGTTTCTTAAAGTTTG-3' | 60°C | 1 min | Figure 1 A 2 | 865 bp |
| | 3 | 5'-CTGAAGGGGATGCTCAATGC-3' | 5'-ATATTTTATATGCTTTTCTTC-3' | 56.1°C | 1 min | Figure 1 A 3 | 850 bp |
| | 4 | 5'-CCAGTCTTAAACTCTAGC-3' | 5'-GGATAATGTCCTCAATCTTTC-3' | 60°C | 1 min | Figure 1 A 4 | 826 bp |
| | 5 | 5'-ACAGCTTCTTAAAGAGATAA AC-3' | 5'-GCCGGAATTATTTTTCTCT-3' | 56.1°C | 1 min | Fig. 1 A 5 | 850 bp |
| | 6 | 5'-GTTTCTCTCAACGCAATTACAA-3' | 5'-ATTTTTTACCTTGCAAGATCC-3' | 60°C | 1 min | Figure 1 A 6 | 873 bp |
| | 7 | 5'-CTGATCTCTGCAGCAAAGACC-3' | 5'-GAAAAACCAATGTTTTTTCTCT-3' | 59.2°C | 1 min | Figure 1A 7 | 908 bp |
| | 8 | 5'-CATTCATAGTGAAGTGATATAA ATCAG-3' | 5'-CTTGCTAAATCCAGAAGAGC-3' | 50.8°C | 1 min | Figure 1 A 8 | 919 bp |
| | 9 | 5'-AGTATCACCTAAAAGATAAGAAG AAC-3' | 5'-CTTTTGAATGGCCTGGTCTTG-3' | 50°C | 1 min | Figure 1 A 9 | 887 bp |
| | 10 | 5'-CCCCCAATTTAAAGAAACTTG-3' | 5'-GCCAATGTATCTCTCCTCC-3' | 53.7°C | 1 min | Figure 1 A 10 | 905 bp |
| | 11 | 5'-CCTCCTGCATGTCTTCTCAAAC-3' | 5'-GGGTATGCTCTTTGAACAACTAC-3' | 60°C | 1 min | Figure 1 A 11 | 886 bp |
| | 12 | 5'-CATGGAACAGAACTGGTAGG-3' | 5'-GTGAAGTAAATAATGTCTTCA-3' | 50°C | 1 min | Figure 1 A 12 | 1094 bp |
| | 13 | 5'-CCTATCCCCATCTAACTGACC-3' | 5'-CAGACACAAGTTGAGTTCTCC-3' | 60°C | 1 min | Figure 1 A 13 | 959 bp |
| | 14 | 5'-GAAGGGCAATCTCCGCACCACG-3' | 5'-CAATCACACTAAGACATCTAAAAAGG-3' | 60°C | 1 min | Figure 1 A 14 | 978 bp |
| For amplification of genomic DNA | exon 1-2 | 5'-GCCGGAATGCTGAGCAGACC-3' | 5'-CTTTTCAAGAACAGCTGAGT-3' | 60°C | 2 min | Figure 1 B 1 | 1193 bp |
| | exon 3 | 5'-CTACAGTCAAAATGTCAAGCG-3' | 5'-GCAATTTGGGAATCTGAAACGAG-3' | 60°C | 1 min | Figure 1 B 2 | 430 bp |
| | exon 4-7 | 5'-ATAAGAATTTAAACCTCCAGAAGATG-3' | 5'-ATTATACCTTCTTCTATATCTTTCAGTC-3' | 60°C | 2 min | Figure 1 B 3 | 1384 bp |
| | exon 8-9 | 5'-GATAATGATTGTCTGACTTGCAGTG-3' | 5'-GGAAAAATCTGATTTTTCTCTTCT-3' | 60°C | 2 min | Figure 1 B 4 | 615 bp |
| | exon 10 | 5'-CTTTAATACGCTTTCAATTTTCGAGATG-3' | 5'-GTCCAGTTTCTAAACCTATATAGC-3' | 60°C | 2 min | Figure 1 B 5 | 1311 bp |
| | exon 11-a | 5'-GTCACCTTTTGTGCTTCTACG-3' | 5'-GGATAATGTCCTCAATCTTTCG-3' | 56.4°C | 2 min | Figure 1 B 6 | 1246 bp |

References:
Yoshikawa et al. BMC Research Notes 2012, 5:173
http://www.biomedcentral.com/1756-0500/5/173
| Exon | Sequence 1 | Sequence 2 | Temperature | Time | Figure |
|------|------------|------------|-------------|------|--------|
| exon 11-b | 5'-ACAGCTTCTAATAAAGAGATAAAAAC-3' | 5'-GCCGGGCATTTATATTITTC-3' | 56.4°C | 1 min | B 7 |
| exon 11-c (same as primer set 6) | 5'-GTTTCTCTCAACGGCAATAACCA-3' | 5'-GATTTTTTTTTTCTCCAGTTCC-3' | 60°C | 1 min | B 8 |
| exon 11-d (same as primer set 7) | 5'-CTGATCTCTGAAGAAGGCC-3' | 5'-GAAAAACCAATGTTTTTTCTCTC-3' | 59.2°C | 1 min | B 9 |
| exon 11-e | 5'-CATTCTAGTGAATGTAATAAATAAACAGAC-3' | 5'-ATTCCCCCTAACACTACATAAAAAG-3' | 56.4°C | 2 min | B 10 |
| exon 12 | 5'-CAATTTTTCTTTTAAATGAGGC-3' | 5'-GAAAAACGTTAGAAAAAGAAACTTAAAAAATAC-3' | 59.2°C | 1 min | B 11 |
| exon 13 | 5'-CTGACCTCTTCTACACTATATTAGTAG-3' | 60°C | 1 min | B 12 |
| exon 14-15 | 5'-CCAAACTTTAAATTTTCTCCTC-3' | 5'-CAGGGATCCCAGTCTATTC-3' | 59.2°C | 2 min | B 13 |
| exon 16 | 5'-GCAGCAAAACATCTGAATGTAG-3' | 5'-GACCTGTGAACCGTAATGTG-3' | 59.2°C | 1 min | B 14 |
| exon 17-8 | 5'-GTTTCTCCAGACGAGATGTTTG-3' | 5'-GAAAAACGTTAGAAAAAGAAACTTAAAAAATAC-3' | 59.2°C | 2 min | B 15 |
| exon 19-20 | 5'-CCATCTAGTGAATGTAATAAATAAACAGAC-3' | 5'-ATTCCCCCTAACACTACATAAAAAG-3' | 59.2°C | 2 min | B 16 |
| exon 21-24 | 5'-CTGACCTCTTCTACACTATATTAGTAG-3' | 60°C | 2 min | B 17 |
| exon 25 | 5'-CTGACCTCTTCTACACTATATTAGTAG-3' | 5'-CCCAATTTTCACAGAAGCCAC-3' | 59.2°C | 1 min | B 18 |
| exon 26 | 5'-GGCTTCTCCATAGTGTAGTAG-3' | 5'-GGACCAACTTGGGGATATTGTC-3' | 59.2°C | 1 min | B 19 |
| exon 27 | 5'-GCTAATTTGCTGATGTTTGTTAC-3' | 5'-GCTGCTGAGTCTCCTAACTCC-3' | 59.2°C | 2 min | B 20 |
| exon 28 | 5'-CTGACCTCTTCTACACTATATTAGTAG-3' | 5'-CCCAATTTTCACAGAAGCCAC-3' | 59.2°C | 1 min | B 21 |
| exon 29 | 5'-GGCTTCTCCATAGTGTAGTAG-3' | 5'-GGACCAACTTGGGGATATTGTC-3' | 59.2°C | 1 min | B 22 |
| exon 30 | 5'-GCTAATTTGCTGATGTTTGTTAC-3' | 5'-GCTGCTGAGTCTCCTAACTCC-3' | 59.2°C | 2 min | B 23 |

For detection of splicing variants

| Exon | Sequence 1 | Sequence 2 | Temperature | Time | Figure |
|------|------------|------------|-------------|------|--------|
| exon 1-11 | 5'-CGAATTTTCTTACACTATTTTGGCAC-3' | 5'-CCCAATTTTCACAGAAGCCAC-3' | 59.2°C | 1 min | B 18 |
| exon 10-14 | 5'-CTGACCTCTTCTACACTATATTAGTAG-3' | 5'-GGACCAACTTGGGGATATTGTC-3' | 59.2°C | 1 min | B 19 |
| exon 10-15 | 5'-CTGACCTCTTCTACACTATATTAGTAG-3' | 5'-GGACCAACTTGGGGATATTGTC-3' | 59.2°C | 1 min | B 20 |

Yoshikawa et al. BMC Research Notes 2012, 5:173
http://www.biomedcentral.com/1756-0500/5/173
Table 1 Nucleotide base sequences of primers (Continued)

| Exon Range          | Forward Primer Sequence | Reverse Primer Sequence | Annealing Temperature | Annealing Duration | PCR Product Size |
|---------------------|-------------------------|-------------------------|-----------------------|-------------------|------------------|
| Exon 11-18          | 5'-CTTCCCTGGGACAATATAG-3' | 5'-GCTGATCTTCTGCTTATC-3' | 50.8°C                | 2 min             | 1417 bp          |
| Exon 15-25          | 5'-CCTCTGACATGTCACAAAC-3' | 5'-GTGTAAGGTTAATAATTTCA-3' | 60°C                  | 2 min             | 1759 bp          |
| Exon 24-27 (same as primer set 13) | 5'-CCTATCCCCAGTTTATCAGCC-3' | 5'-CAGACACAGAATTTGATCTTCC-3' | 60°C                  | 2 min             | 959 bp           |
| For nested PCR of the transcripts lacking most of exon 11 | 5'-GTTCCTACAAATAATATGAATTCCTAACC-3' | 5'-GTTCCTCAGTTGCGAAAG-3' | 58°C                  | 6 min             | 5546 bp and 1506 bp, 1185 bp, 1674 bp or 1270 bp |
| For DNA sequence    |                         |                         |                       |                   |                  |
| cB2 seq1            | 5'-CAATAGAAGGTGTTTCTCCATC-3' |                       |                       |                   |                  |
| cB2 seq2            | 5'-GGATCTCGAGATATTATTATTAG-3' |                       |                       |                   |                  |
| cB2 seq3            | 5'-CAGATCCTGGTCTTTTCTCCATC-3' |                       |                       |                   |                  |
| cB2 seq4            | 5'-CCCCTCTCCACTAAACTGCC-3' |                       |                       |                   |                  |
| cB2 seq5            | 5'-CCTCTCTCTCTACCCATTCG-3' |                       |                       |                   |                  |
| cB2 seq6            | 5'-CCCTCTGAGGAGGCATCGTTC-3' |                       |                       |                   |                  |
| cB2 seq7            | 5'-CCTCTCTCTCTCTCTCTCTCTC-3' |                       |                       |                   |                  |
| cB2 seq8            | 5'-GTCACCAAGGTTGCTCTCTACTC-3' |                       |                       |                   |                  |
| cB2 seq9            | 5'-GCATGCAGAAGTTTCTCTCTCTC-3' |                       |                       |                   |                  |
| cB2 seq10           | 5'-GGATGCCGCAATGTTCTCTCTC-3' |                       |                       |                   |                  |
| cB2 ex14 R (exon)   | 5'-CTAAAGGTTCTTTTTCTTTCA-3' |                       |                       |                   |                  |
| cB2 ex15 F (exon)   | 5'-GCTTTTAAATGTGACATGAG-3' |                       |                       |                   |                  |
| cB2 ex17 R (exon)   | 5'-GTCAGCCAGGGTTGAGTTC-3' |                       |                       |                   |                  |
| cB2 ex18 F (exon)   | 5'-ATATAGTGGAAATTGATACAA-3' |                       |                       |                   |                  |
| cB2 ex22F (exon)    | 5'-CTTTTTAAGGGATCATGACCTG-3' |                       |                       |                   |                  |
| cB2 ex23 F (exon)   | 5'-CCATCACCAGATTTATATCCTCC-3' |                       |                       |                   |                  |
| cB2 ex26 R (exon)   | 5'-CAGAATTTATTTATATATCCCG-3' |                       |                       |                   |                  |
| cB2 ex26 R (exon)   | 5'-CAGAATTTATTTATATCCCG-3' |                       |                       |                   |                  |
To confirm the consensus sequence in miniature Dachshunds, we sequenced BRCA2 DNA from an additional 32 blood samples, and the assembled allele ratio was del:ins = 30:42 (Table 4).

We established a PCR analysis method for canine BRCA2 in order to determine the base sequence from cDNA and genomic DNA, and to detect splicing variants. We identified novel splicing variants in normal canine testes. The functions of these splicing variants were not assessed in this study; nevertheless, these results indicated that the established method was a useful tool for detecting splicing variants.

We also defined the consensus sequence using methods established and described here. During the definition of the consensus BRCA2 ORF, we identified three novel (516 T > C, 2428 T > G, and 8257 T > C) and three reported (1366 T > G, 2609A > C and 4481A > C) single nucleotide variations and two reported insertion/deletion polymorphisms (7126ins/delGTT and 10204ins/delAAA) (Accession numbers: AB622997, AB622998 and AB622999) [11,12,15,18]. The variations 1366 T > G (C386W), 2609A > C (K801Q), 4481A > C (T1425P), and 10204ins/delAAA (M3332IK) are located in the histone acetyltransferase domain, the FANCG binding domain, BRC repeat 3, and nuclear localization signal 2, respectively [13,19-21]. The effects of these variations on BRCA2 function were not understood, with the exception of 10204insAAA; nuclear localization signal 2.

To confirm the consensus sequence in miniature Dachshunds, we sequenced BRCA2 DNA from an additional 32 blood samples, and the assembled allele ratio was del:ins = 30:42 (Table 4).

We established a PCR analysis method for canine BRCA2 in order to determine the base sequence from cDNA and genomic DNA, and to detect splicing variants. We identified novel splicing variants in normal canine testes. The functions of these splicing variants were not assessed in this study; nevertheless, these results indicated that the established method was a useful tool for detecting splicing variants.

We also defined the consensus sequence using methods established and described here. During the definition of the consensus BRCA2 ORF, we identified three novel (516 T > C, 2428 T > G, and 8257 T > C) and three reported (1366 T > G, 2609A > C and 4481A > C) single nucleotide variations and two reported insertion/deletion polymorphisms (7126ins/delGTT and 10204ins/delAAA) (Accession numbers: AB622997, AB622998 and AB622999) [11,12,15,18]. The variations 1366 T > G (C386W), 2609A > C (K801Q), 4481A > C (T1425P), and 10204ins/delAAA (M3332IK) are located in the histone acetyltransferase domain, the FANCG binding domain, BRC repeat 3, and nuclear localization signal 2, respectively [13,19-21]. The effects of these variations on BRCA2 function were not understood, with the exception of 10204insAAA; nuclear localization signal 2.

### Table 2 Comparison between our sequences from the canine BRCA2 open reading frame with registered sequences

| Nucleotide location | 516 T > C | 1366 T > G | 2428 T > G | 2609A > C | 4481A > C | 7126delGTT | 8257 T > C | 10204insAAA |
|---------------------|----------|-----------|-----------|-----------|-----------|-------------|-----------|-------------|
| Amino acid          | 1103T    | C386W     | Silent    | K801Q     | T1425P    | 2307delLL  | Silent    | M3332IK     |
| Coding exon         | 3        | 10        | 11        | 11        | 12        | 18          | 27        |             |
| Novel or reported variation | Novel    | Reported   | Novel     | Reported   | Reported   | Reported    | Novel     | Reported    |
| Present resequencing results | T/C | G/G | T/G | C/A | A/C | ins/del | T/C | ins/ins |
| Full length         | T/T      | G/G       | T/T       | C/C       | A/A       | del/del    | T/T       | ins/ins     |
| Ovary 1             | N.D.     | N.D.      | N.D.      | A.C.      | N.D.      | ins/del    | N.D.      | del/ins     |
| Ovary 2             | T/T      | G/G       | T/T       | C/C       | A/A       | del/del    | T/T       | ins/ins     |
| Testis              | T/T      | G/G       | T/T       | C/C       | A/A       | del/del    | T/T       | ins/ins     |
| Partial Genome      | T.G =    | T.G =     | T.G =     | A.C =     | A.C =     | ins/del    | T.C =     | del/ins     |
|                        | 12:30    | 29:15     | 25:15     | 17:29     |           |             |           |             |
| Registered sequences | Ochii et al. | Testis | T | T | T | T | A | A | ins | T | del |
| Genome project       | Genome   | T | T | T | T | A | A | ins | T | del |
| Bignell et al.       | Genome   | - | - | - | - | T | A | A | - | - | - |
| Hsu et al.           | Mammary gl. | - | - | - | - | T | A | A | - | - | - |
| Total allele frequency | T.C = | T.G = | T.G = | A.C = | A.C = | ins/del = | T.C = | del/ins = |
|                        | 7:1      | 14:36     | 12:1      | 37:20     | 12:1      | 28:20       | 7:1      | 19:35       |
| Consensus sequence   | 516 T | 1366 G | 2428 T | 2609A | 4481A | 7126insGTT | 8257 T | 10204insAAA |
|                        | (103)    | (386 W)   | (740 G)   | (801 K)   | (1425 T)   | (2307insL)  | (2638)   | (3332IK)    |

* N.D., not determined.

a Nucleotide and amino acid location is based on AB043895.5

b Full-length sequence was determined by cDNA sequencing (Accession number; AB622997, AB622998 and AB622999). When frequencies of major and minor alleles were nearly equal or were inconsistent with reported sequences, alleles were further analyzed by partial sequencing of blood genome DNA from 20-23 dogs (Table 3 and 4).

c Sequence from one dog was regarded as one allele because allele type analyses have not been described in these reports. The study by Hsu et al. examined three dogs, while others studied only one dog. Accession numbers for sequences reported by Ochii et al., the Genome Project, Bignell et al. and Hsu et al. are AB043895.5, NC_006607.2, Z75664 and FJ464397-FJ464400, respectively.

### Table 3 Genotype frequency of four variations in normal blood samples

| Nucleotide location | 1366 T > G | 2609A > C | 7126delGTT | 10204insAAA |
|---------------------|------------|-----------|------------|-------------|
| Amino acid          | C386W      | K801Q     | 2307delLL  | M3332IK     |
| Coding exon         | 10         | 11        | 12         | 27          |
| Genotype frequency  | 1366 T homozygosity | 2/21 | 2609A homozygosity | 10/22 | insGTT homozygosity | 9/20 | delAAA homozygosity | 5/23 |
|                     | 1366 G homozygosity | 11/21 | 2609 C homozygosity | 3/22 | delGTT homozygosity | 4/20 | insAAA homozygosity | 11/23 |
|                     | Heterozygosity | 8/21 | Heterozygosity      | 9/22 | Heterozygosity      | 7/20 | Heterozygosity      | 7/23 |

* Nucleotide and amino acid locations are based on AB043895.5
| Sample name | Sex   | Year | Breed            | Nucletide location        | 1366 T > G | 2609A > C | 7126delGTT | 10204insAAA |
|-------------|-------|------|------------------|--------------------------|------------|-----------|------------|-------------|
| K-1         | Male  | 7    | Beagle           | G/G                      | C/C        | del/del   | ins/ins    |             |
| K-2         | Male  | 9    | Labrador retriever | G/G                     | A/C        | ins/del   | ins/ins    |             |
| K-3         | Female| 9    | Mongrel dog      | G/T                      | A/A        | ins/ins   | del/ins    |             |
| K-4         | Male  | 10   | Bichon Frise     | G/T                      | A/A        | ins/ins   | del/ins    |             |
| K-5         | Female| 12   | Pomeranian       | G/G                      | A/A        | ins/ins   | ins/ins    |             |
| K-6         | Female| 12   | Puli             | G/T                      | A/A        | ins/ins   | del/del    |             |
| K-7         | Female| 6    | Puli             | G/T                      | A/A        | ins/ins   | del/ins    |             |
| K-8         | Male  | 10   | Miniature Dachshund | G/T                  | A/C        | ins/del   | del/ins    |             |
| K-9         | Male  | 8    | Miniature Dachshund | G/T                 | A/C        | ins/del   | del/del    |             |
| K-10        | Male  | 8    | Miniature Dachshund | T/T                  | A/A        | ins/ins   | del/del    |             |
| K-11        | Female| 7    | Papillon         | G/G                      | C/C        | del/del   | ins/ins    |             |
| K-12        | Female| 7    | Mongrel dog      | G/G                      | A/A        | ins/ins   | -          |             |
| K-13        | Female| 12   | Miniature Pinscher | T/T                  | A/A        | ins/ins   | del/del    |             |
| K-14        | Male  | 14   | mongrel dog      | G/G                      | A/C        | ins/del   | ins/ins    |             |
| K-15        | Male  | 14   | mongrel dog      | G/G                      | A/A        | -         | ins/ins    |             |
| K-16        | Female| 7    | Papillon         | -                       | C/C        | -         | ins/ins    |             |
| K-17        | Female| 9    | Mongrel dog      | G/G                      | A/A        | ins/ins   | del/ins    |             |
| K-18        | Male  | 10   | Mongrel dog      | G/T                      | A/C        | ins/del   | ins/ins    |             |
| K-19        | Female| 7    | Mongrel dog      | G/G                      | A/C        | -         | ins/ins    |             |
| K-20        | Male  | 10   | Mongrel dog      | G/G                      | A/C        | -         | del/ins    |             |
| K-21        | Male  | 10   | Mongrel dog      | G/G                      | A/C        | -         | del/ins    |             |
| K-22        | Female| 9    | Miniature Dachshund | -                    | A/C        | ins/del   | del/ins    |             |
| K-23        | Male  | 6    | Cavalier King Charles Spaniel | G/T          | -         | del/del   | ins/ins    |             |
| K-24        | Male  | 12   | Mongrel dog      | -                       | -         | del/del   | del/del    |             |
| K-25        | Male  | 10   | Miniature Dachshund | -                    | -         | del/del   | del/del    |             |
| MD-1        | Male  | 10   | Miniature Dachshund | -                    | -         | -         | del/ins    |             |
| MD-2        | Male  | 7    | Miniature Dachshund | -                       | -         | -         | del/ins    |             |
| MD-3        | Male  | 6    | Miniature Dachshund | -                       | -         | -         | ins/ins    |             |
| MD-4        | Male  | 10   | Miniature Dachshund | -                       | -         | -         | del/ins    |             |
| MD-5        | Male  | 12   | Miniature Dachshund | -                       | -         | -         | del/ins    |             |
| MD-6        | Female| 7    | Miniature Dachshund | -                       | -         | -         | del/ins    |             |
| MD-7        | Male  | 12   | Miniature Dachshund | -                       | -         | -         | del/ins    |             |
| MD-8        | Male  | 11   | Miniature Dachshund | -                       | -         | -         | del/ins    |             |
| MD-9        | Female| 9    | Miniature Dachshund | -                       | -         | -         | del/ins    |             |
| MD-10       | Male  | 9    | Miniature Dachshund | -                       | -         | -         | ins/ins    |             |
| MD-11       | Male  | 10   | Miniature Dachshund | -                       | -         | -         | del/ins    |             |
| MD-12       | Female| 14   | Miniature Dachshund | -                       | -         | -         | del/ins    |             |
| MD-13       | Male  | 9    | Miniature Dachshund | -                       | -         | -         | ins/ins    |             |
| MD-14       | Male  | 6    | Miniature Dachshund | -                       | -         | -         | ins/ins    |             |
| MD-15       | Male  | 6    | Miniature Dachshund | -                       | -         | -         | ins/ins    |             |
| MD-16       | Female| 7    | Miniature Dachshund | -                       | -         | -         | del/ins    |             |
| MD-17       | Female| 8    | Miniature Dachshund | -                       | -         | -         | del/ins    |             |
| MD-18       | Male  | 12   | Miniature Dachshund | -                       | -         | -         | del/ins    |             |
| MD-19       | Male  | 10   | Miniature Dachshund | -                       | -         | -         | ins/ins    |             |
| MD-20       | Male  | 6    | Miniature Dachshund | -                       | -         | -         | ins/ins    |             |
| MD-21       | Female| 6    | Miniature Dachshund | -                       | -         | -         | del/ins    |             |
| MD-22       | Male  | 10   | Miniature Dachshund | -                       | -         | -         | ins/ins    |             |
signal 2 harboring the 10204insAAA variation showed enhanced nuclear localization [13]. The other nonsynonymous variations were not located in previously known functional domains.

We identified four variations (1366 T > G, 2609A > C, 7126ins/delGTT, and 10204ins/delAAA), in which the allele frequency of minor variations in genomic DNA from normal blood samples was very high (28.5-37.5%). Such frequent variations in the \textit{BRCA2} gene have not been reported in other species. These highly frequent variations thus appear to be a canine \textit{BRCA2}-specific feature, and should be considered when studying canine \textit{BRCA2}. These four variations were found in the homozygous state in some blood samples from elderly tumor-free dogs. Homozygous mutations in \textit{BRCA2} are assumed to be embryonic-lethal mutations or responsible for Fanconi anemia, which is characterized by bone marrow failure, developmental abnormalities, and predisposition to cancer [22,23]. Thus, these four variations were probably neutral variations, although the 10204insAAA variation is reportedly a candidate malignant mutation in dogs [11].

In this study, we established a PCR analysis method and defined the consensus sequence of \textit{BRCA2} ORF to identify canine \textit{BRCA2} mutations. Using these methods, we are now able to perform \textit{BRCA2} mutation analysis and search for abnormal \textit{BRCA2} splicing variants from mammary tumors in dogs, as is done in human cases.

**Methods**

**Specimens**

Two ovaries (from two mongrel dogs), six testes (from a mongrel dog and five Beagles), a mammary gland (from a female Beagle) and 56 blood samples (Table 4) from tumor-free dogs were kindly provided by Dr. Takashi Kubo and Dr. Go Honda. All experimental procedures were approved by and conducted in accordance with the Guidelines for Institutional Laboratory Animal Care and Use of the School of Veterinary Medicine at Kitasato University, Japan (Approval Number: 11-065).

**Total RNA and genomic DNA extraction, and preparation of cDNA**

Total RNA was isolated from ovaries, six testes, and one mammary gland, which each were stored in RNALater solution (Life Technologies, Grand Island, NY), using a TRIzol and PureLink RNA micro kit (Life Technologies). First-strand cDNA was synthesized from 1-5 μg of total RNA using SuperScript III (Life Technologies). Genomic DNA samples were extracted using a Gentra Puregene tissue kit (Qiagen, Hilden, Germany).

**PCR and sequencing**

For PCR amplification of the full-length canine \textit{BRCA2} ORF from cDNA and all 27 exons from genomic DNA, we designed 14 and 20 primer sets, respectively (Table 1). We also designed five primer sets to detect splicing variants and a primer sets to confirm a novel \textit{BRCA2} transcript that lacked most of exon 11 using nested PCR (Table 1). Each reaction mixture contained 0.1 μL of first-strand cDNA reaction products or 10-50 ng of genomic DNA as a template, each forward and reverse primer at 300 nM, 200 μM dNTPs, 0.02 U of KOD FX DNA polymerase (Toyobo, Japan), and 1× PCR buffer, which was supplied with the enzyme, in a total volume of 10 μL. PCR included one cycle of 2 min at 94°C, followed by 35 cycles of 10 s at 98°C, 30 s at the optimal temperature shown in Table 1 the optimal time shown in Table 1 at 68°C, and a final extension step of 7 min at 68°C. PCR products were treated with shrimp alkaline phosphatase (Affymetrix, Santa Clara, CA) and Exonuclease I (New England BioLabs, Beverly, MA) before sequencing, which was performed with the BigDye Terminator Cycle Sequencing kit Version 3.1 and a ABI PRISM 3100-Avant DNA sequencer (Life Technologies). Direct DNA sequencing was performed at least twice for each amplicon. When we

| Table 4 Information of blood samples and allele type of the four frequently found variations (Continued) |
|---------------------------------------------------------------|
| Sample | Gender | Age (yrs) | Breed | Allele Type | Location |
|-----------------------|------|----------|-------|-------------|----------|
| MD-23 | Female | 10 | Miniature Dachshund | - | - | del/ins |
| MD-24 | Female | 7 | Miniature Dachshund | - | - | del/ins |
| MD-25 | Female | 7 | Miniature Dachshund | - | - | ins/ins |
| MD-26 | Male | 8 | Miniature Dachshund | - | - | ins/ins |
| MD-27 | Female | 9 | Miniature Dachshund | - | - | del/ins |
| MD-28 | Male | 9 | Miniature Dachshund | - | - | ins/ins |
| MD-29 | Male | 8 | Miniature Dachshund | - | - | del/ins |
| MD-30 | Male | 8 | Miniature Dachshund | - | - | del/ins |
| MD-31 | Male | 9 | Miniature Dachshund | - | - | ins/ins |
| MD-32 | Female | 12 | Miniature Dachshund | - | - | del/ins |

*Nucleotide and amino acid locations are based on AB043895.5
-” indicates not determined.
attempted to define the consensus canine BRCA2 ORF sequence, two or three amplicons from each sample were sequenced. Because we detected only three electropherogram patterns among the PCR products with the insertion/deletion mutation sites, we were able to determine the heterozygous insertion/deletion mutations by direct sequencing (Additional file 1: Figure S1).

Additional material

Additional file 1: Figure S1. Example of an electropherogram by direct sequencing from PCR products having the insertion/deletion mutation 7(126ins/delGTT).

Acknowledgements
This work was supported in part by a Kitaato University Research Grant for Young Researchers and a Grant for the Encouragement of Young Scientists from the School of Veterinary Medicine, Kitaato University, Grant-in-Aid for Young Scientists (B) (No. 23780326 and No. 22791476) and for Scientific Research (C) (No. 23580399) from Japan Society for the Promotion of Science.

Author details
1 Laboratory of Veterinary Biochemistry, School of Veterinary Medicine, Kitaato University, Aomori 034-8628, Japan. 2 Division of Disease Model Innovation, Institute for Genetic Medicine, Hokkaido University, Sapporo 060-0815, Japan. 3 Department of Basic Science, School of Veterinary Nursing and Technology, Nippon Veterinary and Life Science University, Tokyo 180-8602, Japan. 4 Department of Small Animal Internal Medicine 2, School of Veterinary Medicine, Kitaato University, Aomori 034-8628, Japan. 5 Department of Veterinary Science, School of Veterinary Medicine, Nippon Veterinary and Life Science University, Tokyo 180-8602, Japan.

Authors’ contributions
YY outlined the design of and coordinated the study, performed the experiments, and drafted the manuscript. MM, K. Ochiai, K. Orino, and WK participated in the design of the study and interpretation of the data and helped to draft the manuscript. K. Okuda, TT, SC, SA, TO, and MB performed several experiments.

Competing interests
The authors declare that they have no competing interests.

Received: 2 February 2012 Accepted: 3 April 2012
Published: 3 April 2012

References
1. De Santis C, Siegel R, Bandi P, Jemal A: Breast cancer statistics, 2011. CA Cancer J Clin 2011, 61(6):409-418.
2. Egenvall A, Bonnett BN, Ohagen P, Olson P, Hedhammar A, von Euler H: Incidence of and survival after mammary tumours in a population of over 80,000 insured female dogs in Sweden from 1995 to 2002. Prev Vet Med 2005, 69(1-2):109-127.
3. Moe L: Population-based incidence of mammary tumours in some dog breeds. J Reprod Fertil Suppl 2001, 57:439-443.
4. Moultou JE, Rosenblatt LS, Goldman M: Mammary tumours in a colony of beagle dogs. Vet Pathol 1986, 23(6):741-749.
5. Gilbertson SR, Kurzman ID, Zachau RE, Huvitz AI, Black MM: Canine mammary epithelial neoplasms: biologic implications of morphologic characteristics assessed in 232 dogs. Vet Pathol 1983, 20(2):127-142.
6. Moultou JE, Taylor DO, Dorn CR, Andersen AC: Canine mammary tumours. Pathol Vet 1970, 7(4):289-320.
7. Evans DG, Shenton A, Woodward E, Laloo F, Howell A, Maher ER: Penetration estimates for BRCA1 and BRCA2 based on genetic testing in a Clinical Cancer Genetics service setting: risks of breast/ovarian cancer quoted should reflect the cancer burden in the family. BMC Cancer 2008, 8:155.
8. King MC, Marks JH, Mandell JB, Group NBYCS: Breast and ovarian cancer risks due to inherited mutations in BRCA1 and BRCA2. Science 2003, 302(5645):643-646.
9. Rivera P, Melin M, Biagi T, Fall T, Haggstroem J, Lindblad-Toh K, von Euler H: Mammary tumor development in dogs is associated with BRCA1 and BRCA2. Cancer Res 2009, 69(22):8770-8774.
10. Rivera P, von Euler H: Molecular biological aspects on canine and human mammary tumours. Vet Pathol 2011, 48(1):132-146.
11. Yoshikawa Y, Morimatsu M, Ochiai K, Nagano M, Tomioka Y, Sasaki N, Hashizume K, Iwanaga T: Novel variations and loss of heterozygosity of BRCA2 identified in a dog with mammary tumors. Am J Vet Res 2008, 69(10):1333-1339.
12. Hsu W-L, Huang Y-H, Chang T-J, Wong M-L, Chang S-C: Single nucleotide variation in exon 11 of canine BRCA2 in healthy and cancerous mammary tissue. Vet J 2010, 184(3):351-356.
13. Yoshikawa Y, Morimatsu M, Ochiai K, Nagano M, Yamane Y, Tomioka N, Sasaki N, Hashizume K: Insertion/deletion polymorphism in the BRCA2 nuclear localization signal. Bioned Res 2005, 263(7):109-116.
14. Ochiai K, Morimatsu M, Tomizawa N, Sutoy B: Cloning and sequencing full length of canine BRCA2 and Rad51 DNA. J Vet Med Sci 2001, 63(10):1103-1108.
15. Borge KS, Barresen-Dale AL, Lingaas F: Identification of genetic variation in 11 candidate genes of canine mammary tumour. Vet Comp Oncol 2011, 9(4):241-250.
16. Sugura T, Matsuyma S, Akyoii H, Takenaka S, Yamate J, Kuxwamura M, Aoki M, Shimada T, Ohashi F, Kubo K: Expression patterns of the BRCA1 and BRCA2 sequence variants in canine normal tissues and mammary gland tumors. J Vet Med Sci 2007, 69(6):587-592.
17. Walker LC, Whitey PJ, Couch FJ, Farugia DJ, Healey S, Eccles DM, Lin F, Butler SA, Goff SA, Thompson BA, et al: Detection of splicing alterations caused by BRCA1 and BRCA2 sequence variants encoding missense substitutions: implications for prediction of pathogenicity. Hum Mutat 2010, 31(6):E1484-1505.
18. Yoshikawa Y, Morimatsu M, Ochiai K, Nagano M, Yamane Y, Tomioka N, Sasaki N, Hashizume K: Analysis of genetic variations in the exon 27 region of the canine BRCA2 locus. J Vet Med Sci 2005, 67(10):1013-1017.
19. Hussain S, Witt E, Huber PAJ, Medhurst AL, Ashworth A, Matthew CG: Direct interaction of the Fanconi anemia protein FANCg with BRCA2/FANCD1. Hum Mol Genet 2003, 12(19):2503-2510.
20. Ochiai K, Yoshikawa Y, Oonuma T, Tomioka Y, Hashizume K, Morimatsu M: Interactions between canine RAD51 and full length or truncated BRCA2 BRC repeats. Vet J 2011, 190(2):293-295.
21. Siddiqui H, Zou JP, Rao VN, Reddy ES: The BRCA2 is a histone acetyltransferase. Oncogene 1998, 16(17):2283-2285.
22. Howlett NG, Taniguchi T, Olson S, Cox B, Waisfisz Q, De Die-Smulders C, Persky N, Grompe M, Joenje H, Pals G, et al: Biallelic inactivation of BRCA2 in Fanconi anemia. Science 2002, 297(5581):606-609.
23. Sharan SK, Morimatsu M, Albrecht U, Lim DS, Regel E, Dinh C, Sands A, Eichele G, Hasty P, Bradley A: Embryonic lethality and radiation hypersensitivity mediated by Rad51 in mice lacking Brca2. Nature 1997, 386(6627):804-810.

doi:10.1186/1756-0500-5-173
Cite this article as: Yoshikawa et al.: Establishment of a PCR analysis method for canine BRCA2. BMC Research Notes 2012 5:173.