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Association of A4304G in exon eleven of Brca2 gene with canine mammary tumour#

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Abstract

Canine mammary tumour is one among the prevalent life style diseases affecting dogs. Certain breeds of dogs are predisposed to mammary neoplasia indicating a possible role of genetic factors in the incidence of disease. Several candidate genes have been studied for its association with mammary tumour in dogs, of which Breast Cancer 2 is one among the genes responsible for the mammary tumour in dogs. In the current study, A4304G within exon 11 of Breast Cancer 2 gene was evaluated for its association with canine mammary tumour. The whole genomic DNA was extracted from 100 female dogs (50 mammary tumour affected dogs and 50 normal animals) above five years. Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (PCR-RFLP) confirmed the presence of the concerned polymorphism among the 100 female dogs under study. On statistical analysis a significant association of the missense variant A4304G with mammary tumour could not be obtained.

Keywords: *Canine mammary tumour, Breast Cancer 2 gene, SNP, PCR-RFLP, association study*.

The growing urbanisation and present situation of Covid 19 pandemic which forced people to stay indoors has resulted in a rise in pet population in India (Morgan *et al*., 2020). Canine mammary tumour (CMT), is one among the frequently reported non-infectious disease in dogs. There exist many genetic and non-genetic factors attributing to CMT. Several studies revealed that *Breast Cancer 2* (*BRCA2*) is a highly penetrant gene in association with breast cancer and CMT. A significant association of germline mutations within *BRCA2* was reported in both benign and malignant CMT (Rivera and Euler, 2011). The exon 11 in canine *BRCA2*, is considered to be a mutation hotspot and a widely studied region in association with CMT. The exon 11 codes

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for the eight BRC repeats which plays very a crucial role in DNA repair mechanism and maintaining genomic stability by binding with RAD51 protein. Yoshikawa *et al.* (2008), in their pioneer study to understand the involvement of *BRCA2* in CMT, reported the involvement of SNP 4304 A→G (K1435R) in mammary tumour affected dogs. In the present study, 4304 A→G was evaluated for its association with CMT.

Materials and methods

Blood samples and data were collected from 50 CMT affected and 50 normal female dogs above five years of age that were presented at Teaching Veterinary Clinical Complex (TVCC), Mannuthy, University Veterinary Hospital, Kokkalai and Government Veterinary Hospitals in and around Thrissur district. The whole genomic DNA was isolated from each blood samples by conventional phenol-chloroform method (Sambrook and Russell, 2001). The concentration and purity DNA were checked by NanoDrop spectrophotometer (Thermo Scientific, USA) and those samples with good quality and concentration were further used for genotyping. Genotyping of the samples were done by PCR-RFLP. Primers (Forward BRCA2_F: ACAGCTAATACGGGGCAAAA and Reverse BRCA2_R: GGTCTTTGCTGCAGGATCA) were designed to amplify a 317 bp product and to detect polymorphism within exon 11 of *BRCA2* from the canine *BRCA2* gene sequence (GenBank Accession No: NC_051829.1).

The PCR conditions were optimised (Table 1) and PCR was done in a Bio-Rad T100™ thermal cycler (USA). The restriction endonuclease which identified the polymorphism was selected from NEB cutter (http://nc2.neb.com/NEBcutter2/). The enzyme *Hpy188III* with recognition site 5'TC$^{\downarrow}$NNGA 3' was used to detect A to G SNP by RFLP. The PCR product (1 µg) was incubated with 5U of *Hpy188III* in buffer (10X) at 37°C for 60 min.

The enzyme digested fragments were assessed in three per cent agarose gels. The representative samples which showed different band patterns were sequenced at Agrigenome Labs Pvt. Ltd. Cochin. The forward and reverse sequences were merged using Emboss merger and the sequences were examined using BLASTn to ensure the match with respective gene sequence. Association of the genotype with the status of CMT was done using Fischer exact method.

Table 1. PCR conditions to amplify 317 bp of exon 11 of *BRCA2*

Stage	Steps	Temperature
I	Initial Denaturation	95°C
II	Denaturation	95°C
	Annealing	59.4°C
	Extension	72°C
III	Final Extension	72°C
Stage 2 repeated for 35 times.		

Results and discussion

Rivera *et al.* (2009) evaluated ten human breast cancer susceptible genes and its association with CMT, and identified *BRCA1* and *BRCA2* as most significant genes in association with CMT. The pioneer report of a polymorphic marker for canine *BRCA2* was done by Yoshikawa *et al*. (2005), where the authors found a single insertion deletion polymorphism namely 10204 *indel* AAA. Similar variation in humans was reported in association with breast cancer (Healey *et al*., 2000, Abdel-Hadi *et al*., 2002 and Sliwinski *et al*., 2005).

The largest exon in canine *BRCA2* is exon 11, which is considered to be the mutation hotspot and a well explored region in association with CMT. The exon 11 codes for the eight BRC repeats, functionally most important region which binds with RAD51 protein and involve in DNA repair mechanism when a double strand break occur. It is involved in cell cycle suppression and inhibition of cell proliferation and thus act as a tumour suppressor gene. Yoshikawa *et al.* (2008) investigated the involvement of *BRCA2* in CMT and reported the involvement of SNP 4304 A→G (K1435R) in mammary tumour affected dogs.

In the current study, the transition A to G at position 4304 within exon 11, resulted in an amino acid change of lysine (K) to arginine (R) at 1435th position of BRCA2 protein was evaluated for its association with CMT by PCR-RFLP. On

conventional PCR, gene fragment of 312bp was amplified (Fig. 1). The amplified fragments, which include the reported SNP A4304G, were further digested using the endonuclease, *Hpy188III*. The restriction enzyme digested products with *Hpy188III* unveiled three different band patterns. (Fig.2) depicting three different genotypes. Representative samples from each genotype were sequenced and three different sequence patterns were identified for genotype AA, AG and GG (Fig. 3, 4 and 5). On analysing sequence similarity of merged sequence using BLASTn, the results showed 99 per cent similarity to *Canis lupus familiaris* Labrador Retriever breed sequence (Sequence ID: KX090066.1) (Fig. 6 and 7).

The population parameters were evaluated using Chi square analysis, which revealed that the variant A4304G were in consistent with Hardy-Weinberg equilibrium at level of significance 0.05. The association of the genotypes with CMT was analysed using Fischer exact method (Table 2). On analysis, among 50 CMT affected dogs, 70 per cent showed AA genotype, 24 per cent showed AG genotype and six per cent showed GG genotype. Similarly, within 50 normal animals, 80 per cent showed AA genotype, 16 per cent showed AG genotype and two per cent showed GG genotype (Table 3). On analysing the status of tumour between the genotypes, it was found

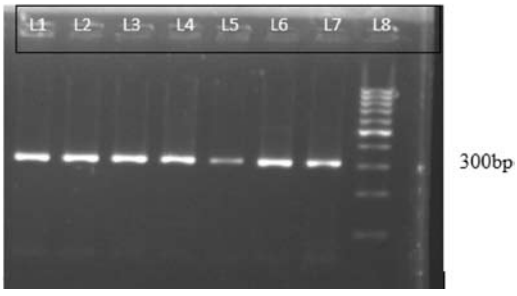

Fig. 1. PCR amplification of *BRCA2* for PCR-RFLP analysis (312 bp fragment). Lane 1-7: 312 bp product Lane 8: 100 bp DNA marker

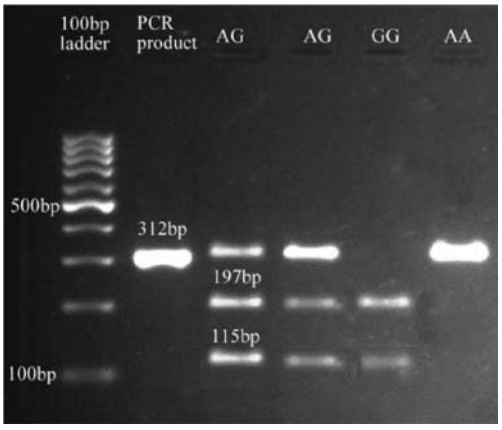

Fig. 2. PCR product and RFLP Pattern in *BRCA2* resolved in three per cent agarose gel

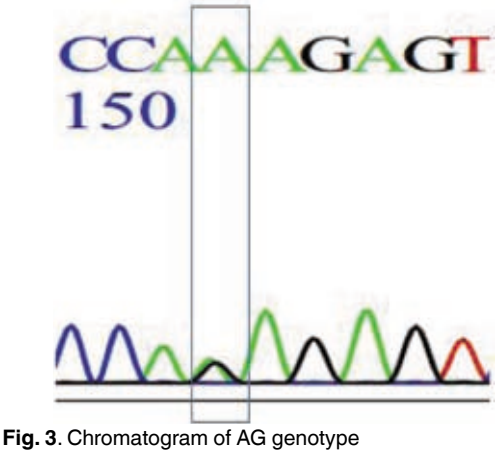

Fig. 3. Chromatogram of AG genotype

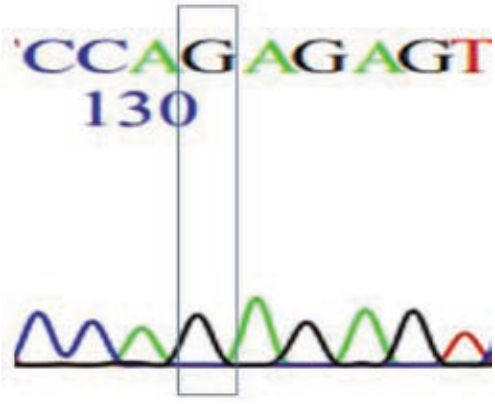

Fig. 4. Chromatogram of GG genotype

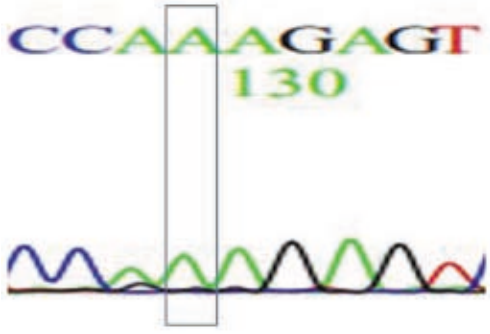

Fig. 5. Chromatogram of AA genotype

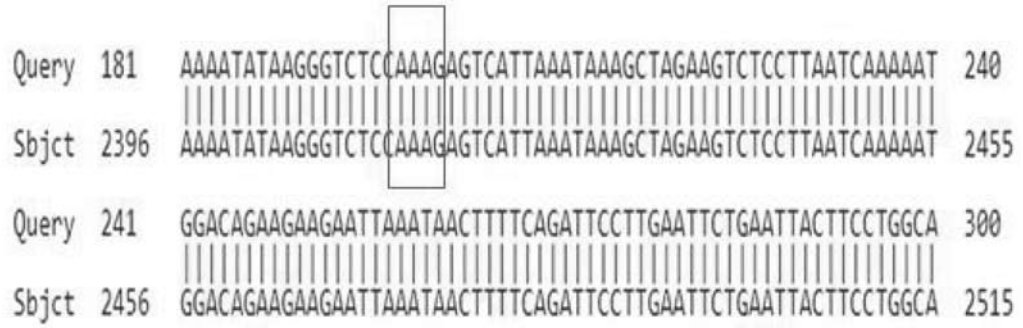

Fig. 6. Sequence analysis using BLASTn

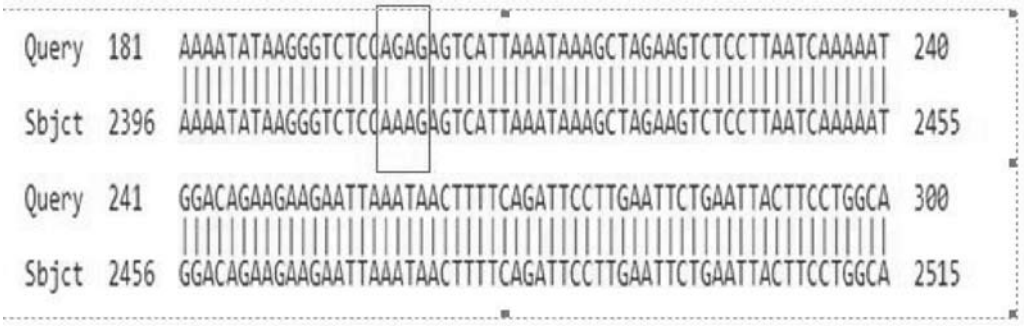

Fig. 7. Sequence analysis using BLASTn showing A to G variation

that within AA genotype, 46.7 per cent were CMT affected and 53.3 per cent were normal animals. Among the AG genotype, 60 per cent were diseased and 40 per cent were normal animals. Similar proportion was obtained within GG genotype, where 60 per cent were affected and 40 per cent were non-affected (Table 4). In the current study a significant association of the considerate polymorphism with CMT could not be obtained.

According to Hsu *et al*. (2010) exon 11 of *BRCA2* can be defined as mutation hotspot for both breast cancer and CMT. They performed polymorphism studies within exon 11 of canine *BRCA2* and identified 19 different SNPs. They observed the variant A4304G, but could not associate the transition with CMT and their findings were in consonance with present study. Huskey *et al*. (2020) performed whole genome sequencing of canine *BRCA2*,

Table 3. Proportion of different genotype within CMT affected and normal animals

Genotypes	AA	AG	GG
Within Tumour (%)	70	24	6
Within Normal (%)	80	16	4

Table 4. Status of CMT within different genotype

	Tumour	Normal
Within AA genotype (%)	46.7	53.3
Within AG genotype (%)	60	40
Within GG genotype (%)	60	40

Table 2. Association analysis of SNP within *BRCA2* with CMT

Genotypes		AA[a]	AG[a]	GG[a]	
TUMOUR	Observed Count	35	12	3	p = 0.569
	Expected Count	37.5	10	2.5	
NORMAL	Observed Count	40	8	2	
	Expected Count	37.5	10	2.5	
Total		75	20	5	

Genotype with same superscript does not differ significantly at p ≤ 0.05

to find out the inherited risk of CMT, where the concerned polymorphism was seen, but could not be associated with the risk of CMT. The reports regarded missense variant K1435R as a variant of unknown significance as far as CMT is considered.

In contrast, Yoshikawa *et al.* (2012) reported that canine K1435R within *BRCA2* were analogues to two mutations namely K1440R and K1440E within human *BRCA2*. These mutations resulted in weaker BRC3–RAD51 interaction, interfering the DNA repair mechanism and leading to tumourogenesis. Maues *et al.* (2018) also detected K1435R in mammary tumour affected dogs. They reported that 68.8 per cent of CMT affected dogs in their study, carried the present mutation.

Certain breeds like English Springer Spaniel, Boxer, Poodle, Bull Mastiff, German Shepherd, Cocker Spaniel, Dachshund and Fox Terrier dogs showed higher risk of incidence of mammary tumour, whereas certain other breeds like Collie, Shetland Sheep dog and Bernese Mountain Dog were considered to be at low risk (Borge *et al*., 2013). Two normal animals with GG genotype in the present study were Poodle (five and half years) and Dachshund (six years) which comes under the high risk group of CMT predisposed breeds. Hence, the absence of statistically significant association of the polymorphism with CMT in the present study, needs to be evaluated further, with modification of sample size present work can be studied for its association.

Conclusion

In the present study, PCR-RFLP analysis of the variant A4304G within exon 11 of *BRCA2* was done and the considerate variant within the population under study was identified. The sequencing results of the patterns obtained revealed the presence of three genotypes AA, AG and GG within the population. But a significant association of the polymorphism with CMT was not obtained. When compared to human breast cancer, molecular studies in CMT were less. So there exists a need for a deeper exploration using more sophisticated molecular technologies like Next Generation Sequencing Techniques in canine mammary tumour. The better understanding of molecular mechanism of canine mammary tumour will help to choose better preventive strategies and therefore bring an enhancement in health and survival of canine population.

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Conflicts of interest

The authors report no conflict of interest.

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