Mammary Tumor Development in Dogs Is Associated with *BRCA1* and *BRCA2*

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Abstract

Breast cancer is a major contributor to overall morbidity and mortality in women. Several genes predisposing to breast cancer have been identified, but the majority of risk factors remain unknown. Even less is known about the inherited risk factors underlying canine mammary tumors (CMT). Clear breed predispositions exist, with 36% of English springer spaniels (ESS) in Sweden being affected. Here, we evaluate 10 human breast cancer genes (*BRCA1, BRCA2, CHEK2, ERBB2, FGFR2, LSP1, MAP3K1, RCAS1, TOX3*, and *TP53*) for association with CMTs. Sixty-three single-nucleotide polymorphisms (SNPs; four to nine SNPs per gene) were genotyped by iPLEX in female ESS dogs, 212 CMT cases and 143 controls. Two genes, *BRCA1* and *BRCA2*, were significantly associated with CMT (Bonferroni corrected $P = 0.005$ and $P = 0.0001$, respectively). Borderline association was seen for *FGFR2*. Benign and malignant cases were also analyzed separately. Those findings supported the association to *BRCA1* and *BRCA2* but with a stronger association to *BRCA1* in malignant cases. Both *BRCA1* and *BRCA2* showed odds ratios of ~4. In conclusion, this study indicates that *BRCA1* and *BRCA2* contribute to the risk of CMT in ESS, suggesting that dogs may serve as a good model for human breast cancer. [Cancer Res 2009;69(22):8770–4]

Introduction

Mammary tumors are the most common neoplasia in intact female dogs (*Canis familiaris*; refs. 1–6). Mammary tumors constitute about half of all tumors in female dogs and approximately half of the canine mammary tumors (CMT) are malignant (7, 8). In both women and dogs, mammary tumors develop with age and they rarely occur before 25 and 5 years of age, respectively (9). The median age of occurrence is 10 to 11 years for dogs; however, some breeds develop CMT at a younger age. The English springer spaniel (ESS) has been shown to have a median age of onset at 6.9 years of age in the Swedish dog population (1). The development of both canine and human mammary tumors is hormone dependent (10, 11). Canine mammary carcinoma have epidemiologic, clinical, morphologic, and prognostic features similar to those of human breast cancer and are therefore suitable models with naturally occurring tumors (12, 13).

The incidence of CMT is 0.05% in female dogs spayed before their first estrus cycle but increases to 8% or 26% if spayed after the first or second heat, respectively (11). If the dog is spayed later than after the second estrus cycle, the risk for malignant tumors is the same as in intact bitches. In Sweden, several spaniel breeds, the doberman, the German shepherd, and the boxer, are predisposed to CMT (1). Breast cancer is often familiar in humans, but a similar hereditary pattern has not been described for mammary tumors in dogs, although breed predilections have been reported (1, 4, 10). Women who have inherited mutations in the *BRCA1* or *BRCA2* (*BRCA1/2*) genes have substantially increased risk of breast cancer, with a lifetime risk of 56% to 84% (14–17). A majority of the *BRCA1/2* mutations reported cause protein truncation through indels, nonsense mutations, splice variants or rearrangements (18–21). A large number of sequence variants with unknown effect on the phenotype have also been detected in *BRCA1/2*, and several studies have tried to determine their clinical significance (22, 23).

Several other genes are also known to confer increased risk for breast cancer in humans (24). The liability for breast cancer is currently believed to be a polygenic trait, where liability is conferred by a large number of loci, each contributing with a small effect on breast cancer risk (25). Four genes, *FGFR2, LSP1, MAP3K1*, and *TOX3*, were recently found to be associated with a mild increase in risk of breast cancer in humans in a genome-wide association study (22). *RCAS1* and *TP53* have been reported to be associated with many types of cancer, including breast cancer (26). *ERBB2* has been shown to have altered expression in human breast cancer, and a deletion in the *CHEK2* gene has been reported as associated with a 2-fold to 3-fold increased risk of breast cancer (27, 28). CMT is also considered a heterogeneous disease with a complex background. It has been suggested that the origin of CMT is multifactorial and depends on an interaction between multiple major and minor genes and environmental factors.

Dogs have a history of inbreeding, which has resulted in low levels of genetic variation within breeds. The recent breed formation and limited population size has also resulted in a high degree of linkage disequilibrium within breeds (29, 30), particularly compared with what is seen in humans (31). Certain breeds are predisposed to specific disorders, and CMT in ESS dogs in Sweden is one such clear example, with 36% of ESS in Sweden being affected by CMT (1). Due to the small genetic variation, CMT should have a more homogenous origin within a single breed compared with breast cancer in the larger human population. This should allow for an easier identification of risk factors within a breed. As part of the dog genome sequencing project, a power calculation for case-control association within dog breeds was performed, suggesting that with 15,000 single-nucleotide polymorphisms (SNPs) the power to detect a locus with a sample of 100 affected and 100 unaffected dogs is 97% for $\lambda = 5$ and 50% for $\lambda = 2$ (30) if

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-09-1725

the frequency of the associated allele is <20%. This supports the notion that, in a genetically isolated population, it is relatively easy to identify a specific founder haplotype, which is significantly more frequent in cases than in controls.

Here we selected 10 genes (*BRCA1, BRCA2, CHEK2, ERBB2, FGFR2, LSP1, MAP3K1, RCAS1, TOX3*, and *TP53*; Table 1) as candidate genes for CMT and performed association using an initial sample set of 89 unrelated cases and 85 unrelated controls and a similar replication set. We found that at least two genes, *BRCA1* and *BRCA2*, were associated with CMT in ESS.

Materials and Methods

Sample collection and DNA isolation. All dogs used in this study were privately owned and registered in the Swedish Kennel Club's database (SKK) with complete pedigrees. The dogs were selected from the databases of Agria pet insurances and SKK, and information was collected regarding possible risk factors for the development of mammary tumors (signalment, age of onset, sex, spaying, lactation, use of contraceptives, diet, pregnancy, disease status, and family cancer history) pathology reports and/or other clinical diagnostic information. All dogs included in the study were female ESS dogs. All the control dogs were older than 8 y, with a confirmed absence of CMT based on palpation of the mammary gland performed by a veterinarian. The dogs were subdivided into two study populations. In the first population (data set 1, n = 192), 100 ESS cases diagnosed with CMT and 92 control dogs were selected. All cases and controls were selected to be unrelated at the parental level. There were 28 cases with malignant tumors, 57 cases with benign tumors, all confirmed with histopathology performed by a veterinary pathologist at one of three central laboratories, and 15 cases where the pathology report was unknown.

In the replication population (data set 2, n = 182), the diagnostic criteria were less stringent and fewer dogs with diagnosed malignant disease were available. One hundred twenty-one ESS cases were selected based on pathology reports if available and, otherwise, based only on physical examination data (the presence of a single or multiple nodules within the mammary gland). Most of these mammary tumors were not surgically excised, or excised and not histopathologically evaluated. Of the 121 cases, 4 were confirmed as malignant and 39 as benign by histopathology. Sixty-one control dogs were available. Siblings were allowed in this population.

Blood samples were collected by veterinarians in different veterinary animal hospitals and veterinary clinics throughout Sweden between the years 2005 and 2009. All sampling of dogs were approved by the owners and conformed to the decision of the Swedish Animal Ethical Committee (no. C9/5) and the Swedish Animal Welfare Agency (no. 30-83/95). DNA was extracted from EDTA blood samples using the QIAamp DNA Blood Mini Kit according to the manufacturer's protocol (Qiagen).

Candidate gene selection and genotyping. Ten genes (*BRCA1, BRCA2, CHEK2, ERBB2, FGFR2, LSP1, MAP3K1, RCAS1, TOX3*, and *TP53*) were selected in the present study as candidate genes for CMT. The samples were genotyped for 63 SNPs using the iPLEX Gold Mass ARRAY according to the manufacturer's protocol (Sequenom). Due to the difference in population structure in humans and dogs, a different SNP selection approach was used here rather than the tagSNP approach, which would have been used in humans based on the human HapMap (32). Within dog breeds there are long haplotypes (~1 Mb in size) resulting from the recent breed creation. This means that most genes reside within a block of complete linkage disequilibrium, where no recombination has occurred since breed creation. Because haplotype maps for individual dog breeds do not exist at this point, it is not possible to pick tagSNPs, but instead one can guess that most of the three to five haplotypes expected to cover a gene would be tagged with at least five SNPs. Thus, the SNPs were chosen from the 2.5 million SNP map described in Lindblad-Toh and colleagues (30). This map has roughly one SNP per thousand bases of sequence and is not exhaustive enough to thoroughly describe coding SNPs. No difference was therefore made between coding and noncoding SNPs. We choose evenly spaced, nonrepetitive SNPs from the start to the end of each gene; we aimed for seven SNPs per gene on average, resulting in four SNPs for some genes with fewer nonrepetitive SNPs reported (e.g., *BRCA2*), to nine SNPs in some large genes (e.g., *FGFR2*). Thus, four to nine SNPs per candidate gene (63 SNPs in total) were selected from the available dog genome sequences in the UCSC Genome Browser, Dog May 2005 (CanFam2) assembly (Table 1 and Supplementary Table S1).

Table 1. Genes evaluated for association to CMT risk

Gene	Human chromosome	Canine chromosome	No. SNPs	Span covered
BRCA2	13	25	4	10.729–10.786 Mb
BRCA1	17	9	8	23.278–23.399 Mb
FGFR2	10	28	9	34.303–34.406 Mb
TOX3	16	2	7	65.949–65.964 Mb
CHEK2	22	26	6	25.089–25.133 Mb
MAP3K1	5	2	6	46.823–46.858 Mb
LSP1	11	18	6	49.138–49.143 Mb
RCAS1	8	13	5	13.125–13.138 Mb
TP53	17	5	7	35.617–35.686 Mb
ERBB2	17	9	5	26.098–26.110 Mb

The primers for amplification and extension were designed using Mass ARRAY Assay Design v.3.1 software. DNA was amplified using PCR, and the remaining nucleotide triphosphates were deactivated by phosphatase treatment (SAP). A single base primer extension step was performed, and the allele specific extension products of different masses were quantitatively analyzed using MALDI TOF Mass Specs.

Data analysis. The primary genotype data was analyzed using the Typer 4.0 Analyzer User Interface software (Sequenom) for cluster analysis. SNPs with a call rate of >75% and a minor allele frequency (MAF) of at least 5% were included in each analysis. Samples with a call rate of ≤75% were excluded from further analysis. After filtering, the number of informative SNPs ranged from 32 to 39 SNPs in the different analyses (Supplementary Table S2).

We analyzed all cases versus all controls for data sets 1 and 2 separately and together to investigate whether a single SNP or haplotype was present at a significantly higher or lower frequency in cases compared with controls and thus associated with CMT. Haplotypes were created from all SNPs remaining after filtering in each gene. Haplotype analysis could not be performed for the *TP53* and *LSP1* genes, because only one SNP remained after filtering for these genes. Malignant versus controls, benign versus controls, and malignant versus benign were analyzed only for affected dogs in data set 1 with diagnosis confirmed by histopathology. Cases in data set 2 were not included due to the low number of cases with confirmed diagnosis by histopathology and to the relatedness of the dogs in this data set. Association analyses for all comparisons were performed with the PLINK software (33) for single χ^2 SNP association, haplotype association, odds ratios, and MAF. Nominal (raw) χ^2 and Bonferroni corrected χ^2 P values were calculated to adjust for the multiple testing that arises from evaluating several SNPs or haplotypes (34, 35). A Bonferroni corrected $P <$ 0.05, with correction for total SNP number remaining after filtering in each analysis, was considered statistically significant, although this likely overcorrects due to the fact that most SNPs within a gene are likely linked to each other and are therefore not unrelated observations.

Results

Association analysis was performed first for data set 1 (100 cases; 28 with malignant tumors, 57 with benign tumors, 15 unclassified, and 92 controls), which contained only unrelated cases and controls. Sixty-three SNPs, selected for even spacing across the candidate genes, were genotyped in the case-control material. In data set 1, 89 cases and 85 controls had a call rate of >75% and were used for further analysis. Nominal single SNP association

Table 2. Association of the best single SNP in each gene to CMT risk

Gene	Best P_{raw} data set 1	Best P_{raw} data set 2	Best P_{raw} total	Best P_{Bonf} total	Odds ratio total	$F_{cases}/F_{controls}$ total
BRCA2	**0.0032**	**6.7×10^{-4}**	**3.9×10^{-6}**	**1.4×10^{-4}**	4.24	0.97:0.88
BRCA1	**0.012**	0.18	**1.3×10^{-4}**	**0.0049**	3.74	0.97:0.91
FGFR2	**0.018**	0.11	**0.0047**	0.18	1.88	0.90:0.83
TOX3	0.29	**0.023**	**0.014**	0.52	1.80	0.92:0.86
CHEK2	0.37	**0.015**	**0.034**	1.0	1.40	0.57:0.49
MAP3K1	**0.025**	0.54	**0.042**	1.0	1.43	0.79:0.72
LSP1	**0.036**	0.64	0.11	1.0	1.45	0.89:0.85
RCAS1	0.25	0.063	0.16	1.0	1.42	0.91:0.88
TP53	**0.010**	0.80	0.20	1.0	1.23	0.38:0.33
ERBB2	0.30	0.33	0.24	1.0	1.45	0.95:0.93
N_{cases}	89	122	212	212	212	212
$N_{controls}$	85	59	143	143	143	143

Abbreviations: P_{raw}, the best χ^2 single SNP P value obtained for each gene [nominal association ($P_{raw} < 0.05$) is indicated in bold]; P_{Bonf}, Bonferroni corrected P values [significant association ($P_{Bonf} < 0.05$) is indicated in bold]; $F_{cases}/F_{controls}$, the risk allele frequency in cases and controls.

was found for six genes: *BRCA1, BRCA2, FGFR2, MAPK1, LSP1*, and *TP53* (Table 2 and Supplementary Table S1). When data set 2 (4 malignant, 39 benign, and 78 unclassified cases and 61 controls) was analyzed, *BRCA2* replicated and *CHEK1* and *TOX3* also reached nominal significance. The *P53* and *LSP1* genes only had one SNP with a MAF of >5% and could therefore not be conclusively studied. When both data sets were combined, *BRCA2* reached the strongest significance ($P_{raw} = 3.9 \times 10^{-6}$ and $P_{Bonf} = 1.4 \times 10^{-4}$) together with *BRCA1* ($P_{raw} = 1.3 \times 10^{-4}$ and $P_{Bonf} = 0.0049$; Table 2). For *BRCA2*, the most significant association was seen for the SNP BICF2G630470214 located in intron 24 of the *BRCA2* gene. The SNP is in a region showing limited levels of conservation and is thus likely not the causative variant. Two SNPs in *BRCA1*, BICF2G630829454 and BICF2G630829457, reached statistical significance. BICF2G630829454 is located within a conserved element in intron 10 of the *BRCA1* gene, whereas BICF2G630829457 is located 3′ of the *BRCA1* gene.

Both *BRCA1* and *BRCA2* had odds ratios of ~4, suggesting the presence of a common predisposing allele with a relative risk of ~4 (Table 2). The *BRCA1* risk allele showed a frequency of 97% in cases and 91% in controls, and the *BRCA2* risk allele a frequency of 97% of cases compared with 88% of the controls, supporting the notion that the risk alleles are indeed very common in the ESS dog breed. The results for all SNPs included in the single SNP association analysis are presented in Supplementary Table S3. Haplotype analysis revealed similar frequencies and P values (Table 3, *BRCA1* $P_{raw} = 1.5 \times 10^{-4}$, *BRCA2* $P_{raw} = 4.8 \times 10^{-6}$). No other genes besides *BRCA1* and *BRCA2* reached significance after Bonferroni correction for multiple testing, although *FGFR2* had a significant nominal P value ($P < 0.005$). The SNP with the strongest association for *FGFR2* is positioned within intron 1 and is not conserved.

To examine if there was a stronger association to any particular gene in the malignant tumors, we used data set 1 where samples were clearly unrelated and had a pathologically validated diagnosis (28 malignant, 57 benign, and 92 controls). When malignant cases and controls were compared, the strongest tentative association was seen to *BRCA1* (Table 4, $P_{raw} = 0.007$ and $P_{Bonf} = 0.27$). A similar association was also seen when malignant cases were compared with the benign cases ($P_{raw} = 0.03$ and $P_{Bonf} = 0.81$).

Discussion

We identified two genes associated with CMT in ESS, *BRCA1* and *BRCA2*. Germ line mutations in *BRCA1* and *BRCA2* are thought to account for 5% to 10% of all breast cancer in women (36, 37), and our results suggest that they also predispose to CMTs based on candidate gene association. In this study, we were able to detect association to risk factors conferring ~4-fold increased risk for both *BRCA1* and *BRCA2* to CMT using data from only 212 cases and 143 controls (Table 2). This is in concordance with previous power calculations stating that canine complex traits can be mapped with a few hundred dogs (30) and shows the advantages of mapping genetic risk factors in dogs compared with humans. However, expanding the cohort in our study could possibly generate significant associations for additional genes, because the disease frequency is as high as ~36% in the ESS breed. This increased disease frequency could either

Table 3. Association of the best haplotypes in each gene to CMT risk

Gene	N_{SNPs}	Best P_{raw}	Best P_{Bonf}
BRCA2	2	**4.8×10^{-6}**	**2.6×10^{-4}**
BRCA1	5	**1.5×10^{-4}**	**0.0082**
FGFR2	7	**0.019**	1.0
TOX3	6	**0.025**	1.0
CHEK2	5	0.070	1.0
MAP3K1	2	0.065	1.0
LSP1	N.A.	N.A.	N.A.
RCAS1	5	0.19	1.0
TP53	N.A.	**N.A.**	N.A.
ERBB2	3	0.20	1.0

NOTE: N_{SNPs}, number of SNPs included in the haplotypes; P_{raw}, the best χ^2 haplotype P value obtained for each gene [nominal association ($P_{raw} < 0.05$) is indicated in bold]; P_{Bonf}, Bonferroni corrected P values [significant association ($P_{Bonf} < 0.05$) is indicated in bold]; N_{cases}, 212; $N_{controls}$, 143.

Table 4. Association of the best single SNP in each gene to malignant and benign tumor risk (data set 1)

Gene	Best P_{raw} malignant versus controls	Best P_{raw} benign versus controls	Best P_{raw} malignant versus benign
BRCA2	**0.020**	0.032	0.97
BRCA1	**0.0068**	0.086	**0.027**
FGFR2	**0.027**	0.068	0.067
TOX3	0.34	0.051	**0.023**
CHEK2	0.60	0.37	0.41
MAP3K1	**0.044**	**0.028**	0.12
LSP1	**0.044**	0.17	0.32
RCAS1	**0.038**	0.25	018
TP53	0.82	**0.036**	0.14
ERBB2	0.75	0.28	0.44
N_{cases}	28	53	26
$N_{controls}$	84	84	50

Abbreviation: P_{raw}, the best χ^2 single SNP *P* value obtained for each gene. Nominal association ($P_{raw} < 0.05$) is indicated in bold. None of the SNPs gives significant *P* values after Bonferroni correction.

be caused by many different risk factors accumulated within the breed or by a few risk alleles of very high frequency. The latter notion is supported by the high frequency (~90%) of both the *BRCA1* and *BRCA2* risk alleles in the healthy ESS dogs. Despite this high allele frequency both risk factors confer a ~4-fold increased risk, suggesting a complex etiology of multiple strong risk factors for this disease.

The polymorphisms showing association in our study are not within coding regions and have unknown function. The most likely scenario is that they tag association signals present over entire *BRCA1* and *BRCA2* or parts of the genes due to the long linkage disequilibrium, and the causative variants are thus to be discovered. Still, the associated SNP present within a noncoding conserved element of intron 10 of the *BRCA1* gene could potentially affect gene regulation and should be evaluated for effects on expression levels of the *BRCA1* transcript. One can also not completely exclude that these associations stem from neighboring genes.

One additional gene, *FGFR2*, showed nominal, but not Bonferroni corrected, association and a 2-fold increased risk together with a high allele frequency (90% in affected, 83% in controls). The *FGFR2* (*fibroblastic growth factor receptor 2*) has been associated to human breast cancer in several studies, but no disease-causing variants have been detected thus far (22, 38, 39). However, a recent study indicates that an intronic SNP in *FGFR2* might alter the function of *FGFR2* and cause the association in several ethnic groups (40). It is possible that also the intronic SNP detected in this study is of functional character, although the fact that it is not conserved makes this less likely. More importantly, additional dogs would possibly yield a significant association also for this gene. Adding further SNP markers in the study could also give a similar effect because several markers were removed from the analysis due to a MAF of <5%. In particular, the *TP53* and *LSP1* gene results would probably benefit from more SNPs being included because only one SNP remained for analysis after MAF filtering. This observation could be caused by a random sampling of uninformative SNPs or could be caused by a low level of diversity within the ESS breed in this region.

Both the *BRCA1* and *BRCA2* genes are part of the granin gene family, mostly functioning as tumor suppressor genes. Both genes are frequently seen together with somatic *p53* mutations (41–45). Whereas the *BRCA1* and *BRCA2* genes belong to the same gene family, the histology of breast cancers in women predisposed by *BRCA1* and *BRCA2* mutations differ in several ways, including the presence on *BRCA2* mutations in male breast cancer and the frequent association of *BRCA1* with ovarian cancer. In addition, *BRCA1* tumors more frequently show a higher grade and are more likely to lack estrogen and progesterone receptors. They are also associated to worse prognosis compared with sporadic cases (46). Less is known about *BRCA2* tumors, but they seem to resemble tumors in sporadic cases to a higher degree (46).

To test if a similar coupling of malignancy could be seen in CMT, we compared the malignant and benign cases. No significant association could be detected (Table 4), but a stronger tentative association for *BRCA1* was found in the malignant cases, whereas *BRCA2* seemed to be equally strongly associated with malignant and benign disease. Due to the limited sample numbers in our study, these results are only preliminary and need further confirmation, but the finding agrees with a report that *BRCA1* nuclear expression is particularly reduced in malignant CMTs (47). Additional samples would help determine if a germ line mutation in *BRCA1* truly is predictive of malignancy in CMT in ESS. However, the presence of *BRCA2* in both groups supports the theory that hyperplastic and benign mammary gland proliferation precedes malignant transformation (48) and have a similar inherited predisposition. This is in concordance with breast cancer development being a molecular continuum from benign disease to actual breast cancer, as has been proposed (49).

In conclusion, the association data obtained in this study indicates that the candidate genes *BRCA1* and *BRCA2* are involved in the development of CMTs. Further studies are necessary to find the actual mutation and to understand the functional mechanism, whereby these genes influence the development and malignancy of this disease in the ESS breed. The study suggests that CMT is an excellent model for human breast cancer, indicating that both humans and dogs can benefit from further comparative studies. A genome-wide association study for CMT is currently in progress and is expected to identify additional strong risk factors.

Disclosure of Potential Conflicts of Interest

The authors have full access to the data and take responsibility for their integrity. The authors declare no commercial associations or conflict of interest and have nothing to disclose.

Acknowledgments

Received 5/11/09; revised 9/2/09; accepted 9/14/09; published OnlineFirst 11/3/09.

Grant support: Thure F. and Karin Forsberg Foundation's Grant, Companion Animal's Research Grant, Agria Pet Insurance Research Foundation, Mikaels Forsgren's fund, the Broad Institute of MIT and Harvard, and the European Commission (LUPA) grant GA-201370. K. Lindblad-Toh is the recipient of a EURYI award from the European Science Foundation.

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We thank the Swedish Kennel Club, Agria Pet Insurance Company, the Swedish breed club of English springer spaniel, the veterinary clinics involved for their support this study, and, especially, all the dog owners who, with great enthusiasm, have participated with their dogs in this study. We also thank Elinor Karlsson, Sarah Fryc, Michele Perloski, Noriko Tonomura, and Ross Swofford for their help and advice.

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