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J Vet Res 67, 427-436, 2023
DOI:10.2478/jvetres-2023-0040

Identification of SNP markers for canine mammary gland tumours in females based on a genome-wide association study – preliminary results

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Received: January 21, 2023 *Accepted: June 28, 2023*

Abstract

Introduction: The development of genetic research over recent decades has enabled the discovery of new genetic markers, such as single nucleotide polymorphisms (SNPs). This, as well as the full sequencing of the dog genome, has enabled genome-wide association studies (GWAS) to be used in the search for genetic causes of canine mammary tumours (CMTs). **Material and Methods:** Genotypic data containing 175,000 SNPs, which had been obtained using the Illumina CanineHD BeadChip microarray technique, were available for analysis in this study. The data concerned 118 bitches, including 36 animals with CMT, representing various breeds and age groups. Statistical analysis was performed in two steps: quality control of genotyping data and genome-wide association analysis based on dominant, recessive, overdominant, codominant, and log-additive models with the single SNP effects. **Results:** A total of 40 different SNPs significantly associated with CMT appearance were detected. Moreover, twelve SNPs showed statistical significance in more than one model. Of all the significant SNPs, two, namely *BICF2G630136001* in the overdominant model and *TIGRP2P107898_rs9044787* in the log-additive model, reached the 5^{-8} significance level. The other SNPs were significant to a 1^{-5} level. **Conclusion:** In the group of SNPs indicated as significant in the GWAS analysis, several transpired to be localised within genes that may play an important role in CMT.

Keywords: dogs, canine mammary tumours, genetic marker, genome-wide association studies, SNP.

Introduction

Neoplastic diseases are a serious problem not only in human medicine, but also in veterinary medicine of companion animals. In dogs especially, the incidence of neoplastic diseases seems to be increasing. It is most likely related to the extension of domestic animal life, more accurate diagnostic tools, and the exposure of both humans and animals to the same carcinogens due to similar living and environmental conditions. The sequencing of the genome of the domestic dog (*Canis lupus familiaris*) in 2005 (31) was not only a breakthrough in research on this species, but also established the dog as a model organism for comparative oncological research on the genetic basis of humans. This owes all to the high similarity between the canine and human genomes, amounting to approximately 80%, as well as to the similar aetiopathogenesis of certain types of cancer in both dogs and humans, including mammary gland tumours (38).

Mammary gland neoplasms in dogs are a very interesting and extremely important research model, as they account for nearly 14% of all neoplastic lesions in this species and are second only to skin tumours in terms of incidence (11). In histopathological assessments, approximately 40–50% of mammary gland tumours are malignant neoplasms, most of which are of epithelial origin (29). These tumours are the most common type of neoplasm in bitches, occurring more frequently (the incidence rate is 162–198 cases per 100,000 dogs per year) than in other animal species and three times more often than in women (15, 37). In addition to sex, factors that may be associated with the occurrence of mammary gland cancer include age (37), breed (15), physical condition (22) and hormonal exposure (6).

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The development of genetic research over the last few decades has made it possible to discover new genetic markers such as single nucleotide polymorphisms (SNPs), to which are mainly attributed genetic variability between individuals. Single nucleotide polymorphisms may also contribute to changes in gene expression, which is why they are considered potential markers of carcinogenesis, and are therefore a valuable tool in the early diagnosis of various types of cancer (13).

Unfortunately, veterinary oncogenomics is not developing as dynamically as human medical oncogenomics. The first cancer genome-wide association study (GWAS) in dogs was published by Shearin *et al.* (36), and it showed that 96% of the dogs affected with histiocytic sarcoma shared the same primary locus, featuring a single haplotype spanning the *MTAP* and a part of the *CDKN2A* genes. It was also discovered that this haplotype is within the chromosome region homologous to human chromosome (chr) 9p21, which has been found to correlate with several types of cancer. A study by Karyadi *et al.* (20) using the GWAS method identified two loci (*KITLG* and *MC1R*) associated with the risk of finger squamous cell carcinoma in poodles. A similar finding using GWAS was published by Karlsson *et al.* (19), who studied three breeds at high risk of canine osteosarcoma, two being sighthounds (the racing greyhound and the Irish wolfhound) and the third a distantly related breed (the Rottweiler). Their research confirmed that the *CDKN2A*/*B* gene is associated with the development of osteosarcoma in dogs.

The first GWAS to identify the genetic basis of canine mammary tumours (CMT), conducted on 332 English springer spaniels, showed eight statistically significant SNPs in two sets of four each, one set on chromosome 11 and the other on chromosome 27 (27). The most significant genome-wide associations were detected for SNP *BICF2G630310626* (chromosome 11; 73,290,522 base pairs), which is located in a region containing the regulator of cyclin-dependent kinase 5 (*CDK5RAP2*). This corresponds to the results of Karlsson *et al.* (19), who identified cyclin-dependent kinase (CDKN2A) as a potential oncofactor in an independent GWAS study on osteosarcoma, indicating an important role of these proteins in cancer development.

Only a few studies to date have used the GWAS method to look at the association between specific SNPs and the risk of mammary gland cancers in dogs. Most studies by other authors (6, 7, 32, 35) have focused only on analysing the relationship between specific SNP variants in genes commonly known to be associated with increased susceptibility to breast cancer in humans. Canadas *et al.* (5) conducted research in this area, and showed 67 SNPs that may be related to the occurrence of CMT. However, they analysed only 14 such genes: the proto-oncogenes *HER2* and *EGFR*; the tumour suppressor genes *TP53* and *STK11*; the DNA damage recognition and repair genes *BRCA1*, *BRCA2*, *BRIP*, *CHEK2*, *PTEN* and *RAD51*; and the hormonal metabolism genes *ESR1*, *COMT*, *PGR* and *PRLR*.

Despite the research outlined above, the genetic basis of CMT is still poorly understood compared to breast cancer in humans, as evidenced by the small number of SNPs found for individual genes that may be associated with the risk of tumour occurrence (23). Therefore, the aim of the study was to identify SNPs associated with the occurrence of CMT in bitches based on GWAS data.

Material and Methods

Material. One-hundred and eighteen unrelated bitches of different breeds (primarily golden retrievers, Labrador retrievers, Yorkshire terriers, German shepherds, French bulldogs, and Maltese) and of mixed breed, aged 5 months to 16.5 years (mean 5.8 years) were included in the analysis. Of these, 36 had mammary gland tumours confirmed by histopathological examination (14) of the material collected by veterinarians following lesion removal.

DNA isolation and genotyping. Four types of material were used for DNA isolation: whole venous blood and tissue obtained during sterilisation (in the case of healthy dogs) and whole venous blood neoplastic tissue obtained from the tumour removal procedures (in the case of bitches with a tumour). All samples were sent to the Polish Federation of Cattle Breeders and Dairy Farmers (Warsaw, Poland), where total DNA was isolated from them with a Sherlock AX kit (A&A Biotechnology, Gdansk, Poland), according to the manufacturer's protocol (1). After isolation, quantitative evaluation was performed using a NanoDrop2000P spectrophotometer (Thermo Scientific, Waltham, MA, USA), which was the means for both the purity (A_{260}/A_{280}) and the concentration (ng/μL) of the DNA to be determined. Genotyping was performed using the CanineHD BeadChip microarray (175,000 SNPs) (Illumina, San Diego, CA, USA).

Statistical analysis. Statistical analysis was performed in two steps, namely quality control of genotyping data and GWAS based on statistical models with the single SNP effects. First, SNPs with a large number of missing observations were excluded from the dataset. It was assumed that the lower limit of completeness was 95% and markers for which the number of misses reduced completeness to a lower percentage did not take part in further analysis (17). Next, the individuals with a call rate not exceeding 90% were also excluded (27). Another SNP selection criterion was minor allele frequency (MAF), for which a 5% threshold was used (25). Typing with SNPs with low MAF may lead to incorrect detection of phenotypic associations and may also be more prone to genotyping errors. The last selection criterion was the exclusion of markers deviating from the Hardy–Weinberg equilibrium. Marker SNPs for which the P-value of the test for compliance with the theoretical equilibrium frequencies did not exceed 1^{-10} in the case group and 1^{-6} in the control group were removed. The SNPs selected by the above criteria were used for further analyses.

The association analysis was based on logistic regression models with a single SNP marker as the explanatory variable. The analytical model was in the general form of:

$$\log\left(\frac{\pi}{1-\pi}\right) = \alpha + \beta g + \gamma Z$$

where π is the probability of being a case; α, β and γ are estimated model parameters; g is the polymorphism, which will encode three different genotypes, and Z is the matrix of non-genetic factors influencing the probability of being a case.

This approach makes the use possible of different types of genetic models (codominant, dominant, recessive, overdominant and log-additive) and was implemented using the SNPassoc package (16, 18) in R (33). The statistical significance of an association between the SNP and the analysed phenotype was determined on the basis of P-values for the likelihood ratio test. For each SNP, different genetic models were compared in terms of the Akaike criterion. For each genetic model and SNP for which a significant association was detected, the numbers and percentages of analysed SNP genotypes and odds ratios with 95% confidence intervals were calculated.

Results

Single-nucleotide polymorphisms with missing observations (<geno 95%), low MAF (5%) or deviating from the Hardy–Weinberg equilibrium (HWE) were excluded from the dataset (Fig. 1). This selection criteria reduced the number of SNPs from 173,662 to 140,672 (by 19%). Single-nucleotide polymorphisms within individual chromosomes ranged from 1,938 for chromosome 38 to 9,006 for chromosome 1. Selection using these criteria resulted in association analyses using from 70.5% of SNPs for the X chromosome to 86.8% of SNPs for chromosome 35.

To verify the statistical significance of the relationship between individual SNPs and the appearance of a mammary gland tumour, the dominant, recessive, codominant, overdominant, and log-additive models were used based on logistic regression (Table 1). A total of 40 different SNPs with a statistically significant effect on mammary gland tumour appearance were detected. Twelve SNPs (*BICF2P448058*, *BICF2P501513*, *BICF2P776685* (chr1), *BICF2G630704243* (chr3), *TIGRP2P107898_rs9044787* (chr8), *TIGRP2P176993_rs9197628* (chr13), *BICF2P797481* (chr14), *BICF2P1314057* (chr16), *TIGRP2P268994_rs8894778* (chr19), *BICF2P373712* (chr23), *BICF2S23049081* (chr34) and *BICF2G630136001* (chr37)) demonstrated statistical significance for more than one model, and therefore the table displays a total of 56 SNPs. Two of the significant SNPs – *BICF2G630136001* using the overdominant model and *TIGRP2P107898_rs9044787* using the log-additive model – reached a significance level of 5^{-8}.

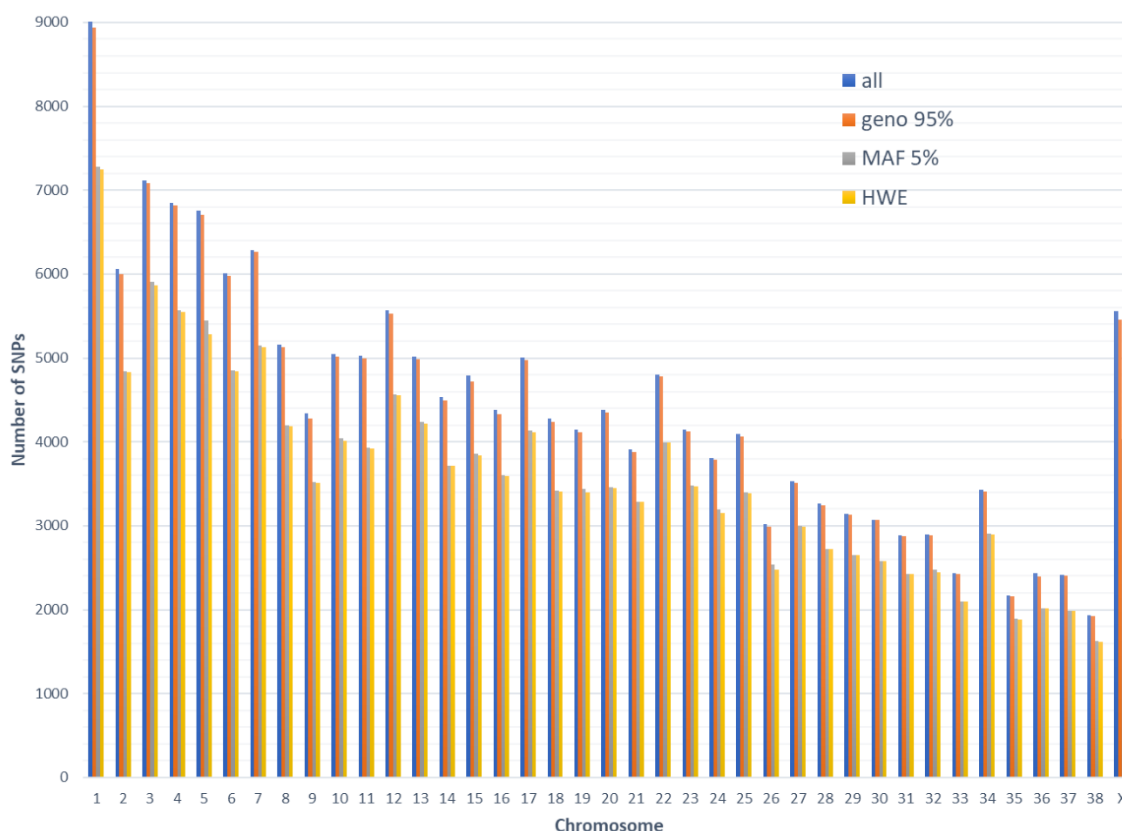

Fig. 1. Single nucleotide polymorphism (SNP) selection results
geno 95% – SNPs for which the number of missing observations did not exceed 5%; MAF – minor allele frequency; MAF 5% – SNPs for which the frequency of the rarer allele was greater than 5%; HWE – Hardy–Weinberg equilibrium (SNP for which the genetic balance criterion was met)

Table 1. Single nucleotide polymorphisms statistically significantly associated with the occurrence of mammary gland tumours

Chromosome	Codominant	Dominant	Recessive	Overdominant	Log-additive
1	*BICF2P448058* *BICF2P501513* *BICF2P776685*	*BICF2P448058* *BICF2P501513*	*BICF2S2334324*	*BICF2P343250*	*BICF2P825735* *BICF2P501513* *BICF2P776685* *BICF2P345133*
2	-	*TIGRP2P33447_rs8841788*	-	-	-
3	-	*BICF2G630704243*	*BICF2S23125394*	-	*BICF2G630704243* *BICF2G630704438*
8	*TIGRP2P107898_rs9044787*	*BICF2S2308912*	*TIGRP2P107898_rs9044787*	*BICF2P158200*	*TIGRP2P107898_rs9044787**
10	-	-	-	*BICF2S23760334*	-
11	-	-	*BICF2P576198*	*BICF2S23118240*	-
12	-	*BICF2S23638049*	-	-	-
13	-	*TIGRP2P176993_rs9197628*	-	-	*TIGRP2P176993_rs9197628*
14	*BICF2P797481*	-	-	-	*BICF2P797481* *BICF2P720053*
15	-	*TIGRP2P201649_rs8752112* *BICF2S23334099* *BICF2P352914*	-	-	-
16	*BICF2P1314057*	*BICF2P1314057*	-	-	*BICF2P1314057*
19	*TIGRP2P268994_rs8894778*	-	-	*TIGRP2P268994_rs8894778*	-
22	-	-	*BICF2P801296*	-	-
23	*BICF2P373712*	*BICF2P373712*	-	-	*BICF2P373712*
24	-	-	-	-	*BICF2S23648284*
28	-	*BICF2G630269882*	-	-	-
30	-	-	-	-	*BICF2G630397948*
32	-	*BICF2S23661944*	-	-	-
34	-	-	*BICF2S23049081*	-	*BICF2S23049081*
35	-	-	*BICF2S23219997*	-	*BICF2P28560*
37	*BICF2G630136001*	-	-	*BICF2G630129768* *BICF2G630136001**	*BICF2G630127550*
X	-	-	-	*BICF2S23347259*	-

The effect of single nucleotide polymorphisms marked with * was statistically significant at the level 5^{-8}

Significant SNPs; their location and position in base pairs; minor alleles with their overall, in-control-group and in-case-group frequencies; HWE p-values; and candidate gene or locus, or the closest neighbourhood gene or locus are shown in Table 2. The overall minor allele frequency ranged from 14.83% for *BICF2P825735* to 49.58% for *BICF2S23049081*. In some cases (*BICF2P448058*, *BICF2P501513*, *BICF2P345133*, *TIGRP2P33447_rs8841788*, *TIGRP2P107898_rs9044787*, *BICF2G630397948* and *BICF2S23049081*) the minor allele turned out to be the major one for the group of cases. Conversely, some minor alleles were the major ones in the control group (*BICF2P776685*, *BICF2S2334324*, *BICF2G630704243*, *BICF2S23125394*, *BICF2G630704438*, *BICF2P576198*, *BICF2P797481*, *BICF2P720053*, *BICF2P801296* and *BICF2P373712*). Of the 40 analysed SNPs, 24 passed the HWE compliance test at a significance level of $\alpha = 0.05$. The frequencies of alleles within the remaining markers were not consistent with the HWE, but it should be noted that all of them met the SNP selection criteria, and the zero values appearing in the table are the result of rounding.

Odds ratios were calculated to illustrate the risk of mammary gland tumours associated with particular genotypes within particular SNPs and are presented in Tables 3–7. Analysis based on the dominant model (Table 3) showed that the presence of a minor allele in 7 of the 14 SNPs, either in the form of a homozygote or heterozygote, increased the likelihood of developing the disease. The odds ratio ranged from 6.76 for *TIGRP2P176993_rs9197628* to 24.79 for *BICF2P448058*. In the remaining 7 SNPs, the presence of a minor allele was associated with a decrease in the probability of developing cancer. The odds ratios ranged from 0.04 for *BICF2S2308912* and *BICF2S23661944* to 0.15 for *BICF2G630704243*.

The recessive model (Table 4) showed an increase in the probability of developing a mammary gland tumour in the case of a minor allele homozygous for three markers, namely *TIGRP2P107898_rs9044787* (OR = 17.22), *BICF2S23049081* (OR = 7.43) and *BICF2S23219997* (OR = 31.15). A decrease in the probability of tumour appearance in the case of minor allele homozygotes was noted for *BICF2S2334324* (OR = 0), *BICF2S23125394* (OR = 0.04), *BICF2P576198* (OR = 0.04) and *BICF2P801296* (OR = 0.05).

Table 2. Summary of single nucleotide polymorphisms (SNPs) statistically significantly associated with the occurrence of mammary gland tumours

SNP	CHR	Location (bp)	Minor allele	MAF (%)	$MAF_{control}$ (%)	MAF_{case} (%)	HWE P-value	Candidate gene or locus/nearest gene or locus
BICF2P448058	1	65,212,166	G	46.19	37.20	66.67	0.85	*LOC111096229 / TRMT11*
BICF2P501513	1	65,293,673	A	42.37	32.93	63.89	1.00	*- / LOC111096229, LOC111096230*
BICF2P776685	1	65,819,801	A	46.61	56.71	23.61	0.46	*- / LOC100684456, LOC102153070*
BICF2S2334324	1	14,217,295	G	47.03	52.44	34.72	0.47	*ZCCHC2 / -*
BICF2P343250	1	67,922,585	A	27.97	32.32	18.06	0.25	*LAMA2 / -*
BICF2P825735	1	41,988,950	A	14.83	6.71	33.33	0.00	*ARMT1*
BICF2P345133	1	66,185,882	A	36.86	26.22	61.11	0.00	*- / LOC111096246*
TIGRP2P33447_rs8841788	2	82,135,437	G	39.32	32.93	54.29	0.70	*ARHGEF19 / -*
BICF2G630704243	3	27,421,551	G	41.53	51.22	19.44	0.34	*HOMER1 / -*
BICF2S23125394	3	36,857,983	A	49.57	57.93	30.00	0.06	*- / MKRN3*
BICF2G630704438	3	27,777,144	A	49.57	59.76	26.39	0.03	*DMGDH / -*
TIGRP2P107898_rs9044787	8	12664933	A	36.75	24.07	65.28	0.05	*NPAS3 / -*
BICF2S2308912	8	49,696,532	A	19.07	26.83	1.39	0.00	*- / LOC111097115*
BICF2P158200	8	34,943,508	A	17.37	11.59	30.56	0.34	*CCDC175, JKAMP / -*
BICF2P441276	8	56,964,583	A	39.57	37.80	43.94	0.02	*LOC111097128 / FLRT2*
BICF2S23760334	10	47,442,988	A	36.44	33.53	43.06	0.00	*- / LOC100687001, PPM1B*
BICF2P576198	11	61,345,663	G	47.88	56.71	27.78	0.01	*- / LOC102156133, LOC612266, SMC2*
BICF2S23118240	11	52,671,467	A	23.73	26.22	18.06	0.01	*- / DNAJB5, C11H9orf131*
BICF2S23638049	12	19,343,255	A	26.50	19.51	42.86	1.00	*- / LOC119876904, LOC119874254*
TIGRP2P176993_rs9197628	13	39,050,399	A	19.49	10.98	38.89	0.38	*LIMCH1 / -*
BICF2P797481	14	45,742,638	A	42.80	53.05	19.44	0.19	*- / LOC106559680*
BICF2P720053	14	45,744,668	G	42.37	52.44	19.44	0.19	*- / LOC106559680*
TIGRP2P201649_rs8752112	15	42,346,461	A	27.12	35.37	8.33	0.00	*LOC119869373 / ASCL1*
BICF2P352914	15	42,358,113	G	27.12	35.37	8.33	0.00	*ASCL1 / -*
BICF2S23334099	15	42,352,911	A	27.54	35.98	8.33	0.00	*ASCL1 / -*
BICF2P1314057	16	35,869,183	A	23.28	14.63	44.12	1.00	*RBPMS / -*
TIGRP2P268994_rs8894778	19	46,961,958	A	27.35	22.56	38.57	0.06	*ARHGAP15 / -*
BICF2P801296	22	7,706,835	A	49.15	56.10	32.86	0.10	*ENOX1 / -*
BICF2P373712	23	51,871,689	G	43.53	55.63	16.67	0.00	*- / LOC111092021, VEPH1*
BICF2S23648284	24	29,281,559	T	25.42	17.07	44.44	1.00	*LOC119865549 / -*
BICF2G630269882	28	20,841,387	A	29.91	21.95	48.57	1.00	*- / LOC111092968*
BICF2G630397948	30	35,183,072	A	42.37	31.71	66.67	0.00	*THSD4 / -*
BICF2S23661944	32	29,141,932	A	16.53	23.17	1.39	0.09	*LOC106558120 / -*
BICF2S23049081	34	30,205,708	G	49.58	39.02	73.61	0.07	*- / LOC111093899*
BICF2S23219997	35	20,654,940	A	22.03	14.63	38.89	0.01	*- / LOC119867672*
BICF2P28560	35	15,603,710	A	36.44	46.34	13.89	0.05	*ATXN1 / -*
BICF2G630136001	37	30,735,796	A	38.46	34.76	47.14	0.56	*DLGAP2 / -*
BICF2G630129768	37	24,240,716	G	38.98	35.98	45.83	0.44	*- / LOC102156106, LOC111094390*
BICF2G630127550	37	9,335,944	G	40.25	50.00	18.06	0.03	*SPATS2L / -*
BICF2S23347259	X	32,408,330	A	31.62	25.61	45.71	0.20	*LANCL3 / -*

CHR – chromosome; MAF – minor allele frequency; $MAF_{control}$ – minor allele frequency in the control group; MAF_{case} – minor allele frequency in the case group; HWE – Hardy–Weinberg equilibrium

Table 3. The risk of mammary gland tumours associated with particular genotypes within single nucleotide polymorphisms (SNPs) for the dominant model

CHR	SNP	Genotype	Genotype number (percentage)		OR (95% CI)	P-value
			Control	Case		
1	*BICF2P448058*	A/A G/A-G/G	34 (41.5%) 48 (58.5)	1 (2.5%) 35 (97.2%)	24.79 (3.24; 189.80)	1.57×10^{-6}
	BICF2P501513	G/G A/G-A/A	37 (45.1%) 45 (54.9%)	2 (5.6%) 34 (94.4%)	13.98 (3.15; 62.08)	7.08×10^{-6}
2	*TIGRP2P33447_rs8841788*	A/A G/A-G/G	41 (50.0%) 41 (50.0%)	3 (8.6%) 32 (91.4%)	10.67 (3.03; 37.61)	5.15×10^{-6}
3	*BICF2G630704243*	A/A G/A-G/G	19 (23.2%) 63 (76.8%)	24 (66.7%) 12 (33.3%)	0.15 (0.06; 0.36)	7.02×10^{-6}
8	*BICF2S2308912*	G/G A/G-A/A	49 (59.8%) 33 (40.2%)	35 (97.2%) 1 (2.8%)	0.04 (0.01; 0.33)	2.67×10^{-6}
12	*BICF2S23638049*	G/G A/G-A/A	55 (67.1%) 27 (32.9%)	8 (22.9%) 27 (77.1%)	6.87 (2.76; 17.14)	7.92×10^{-6}
13	*TIGRP2P176993_rs9197628*	G/G A/G-A/A	65 (79.3%) 17 (20.7%)	13 (36.1%) 23 (63.9%)	6.76 (2.85; 16.06)	6.52×10^{-6}
	TIGRP2P201649_rs8752112	G/G A/G-A/A	38 (46.3%) 44 (53.7%)	32 (88.9%) 4 (11.1%)	0.11 (0.03; 0.33)	4.35×10^{-6}
15	*BICF2S23334099*	G/G A/G-A/A	37 (45.1%) 45 (54.9%)	32 (88.9%) 4 (11.1%)	0.10 (0.03; 0.32)	2.50×10^{-6}
	BICF2P352914	A/A G/A-G/G	38 (46.3%) 44 (53.7%)	32 (88.9%) 4 (11.1%)	0.11 (0.03; 0.33)	4.35×10^{-6}
16	*BICF2P1314057*	G/G A/G-A/A	60 (73.2%) 22 (26.8%)	8 (23.5%) 26 (76.5%)	8.86 (3.49; 22.48)	6.14×10^{-7}
23	*BICF2P373712*	A/A G/A-G/G	21 (26.2%) 59 (73.8%)	26 (72.2%) 10 (27.8%)	0.14 (0.06; 0.33)	2.77×10^{-6}
28	*BICF2G630269882*	C/C A/C-A/A	51 (62.2%) 31 (37.8%)	6 (17.1%) 29 (82.9%)	7.95 (2.97; 21.31)	3.93×10^{-6}
32	*BICF2S23661944*	G/G A/G-A/A	50 (61.0%) 32 (39.0%)	35 (97.2%) 1 (2.8%)	0.04 (0.01; 0.34)	4.52×10^{-6}

CHR – chromosome; OR – odds ratio; CI – confidence interval

Table 4. The risk of mammary gland tumours associated with particular genotypes within single nucleotide polymorphisms (SNPs) for the recessive model

CHR	SNP	Genotype	Genotype number (percentage)		OR (95% CI)	P-value
			Control	Case		
1	*BICF2S2334324*	A/A-G/A G/G	54 (65.9%) 28 (34.1%)	36 (100%) 0 (0%)	0 0	9.24×10^{-6}
3	*BICF2S23125394*	C/C-A/C A/A	49 (59.8%) 33 (40.2%)	34 (97.1%) 1 (2.9%)	0.04 (0.01; 0.33)	3.70×10^{-6}
8	*TIGRP2P107898_rs9044787*	G/G-A/G A/A	77 (95.1%) 4 (4.9%)	19 (52.8%) 17 (47.2%)	17.22 (5.19; 57.15)	9.55×10^{-8}
11	*BICF2P576198*	A/A-G/A G/G	49 (59.8%) 33 (40.2%)	35 (97.2%) 1 (2.8%)	0.04 (0.01; 0.33)	2.67×10^{-6}
22	*BICF2P801296*	G/G-A/G A/A	50 (61.0%) 32 (39.0%)	34 (97.1%) 1 (2.9%)	0.05 (0.01; 0.35)	6.19×10^{-6}
34	*BICF2S23049081*	A/A-G/A G/G	69 (84.1%) 13 (15.9%)	15 (41.7%) 21 (58.3%)	7.43 (3.05; 18.08)	4.35×10^{-6}
35	*BICF2S23219997*	G/G-A/G A/A	81 (98.8%) 1 (1.2%)	26 (72.2%) (27.8%)	31.15 (3.81; 255.00)	8.60×10^{-6}

CHR – chromosome; OR – odds ratio; CI – confidence interval

Table 5. The risk of mammary gland tumours associated with particular genotypes within single nucleotide polymorphisms (SNPs) for the codominant model

CHR	SNP	Genotype	Genotype number (percentage)		OR	95% CI	P-value
			Control	Case			
1	*BICF2P448058*	A/A	34 (41.5%)	1 (2.8%)	1.00		
		G/A	35 (42.7%)	22 (61.1%)	2.73	(2.73; 167.5)	6.1×10^{-6}
		G/G	13 (15.9%)	13 (36.1%)	4.03	(4.03; 286.7)	
	BICF2P501513	G/G	37 (45.1%)	2 (5.6%)	1.00		
		A/G	36 (43.9%)	22 (61.1%)	11.31	(2.48; 51.61)	7.1×10^{-6}
		A/A	9 (11.0%)	12 (33.3%)	24.67	(4.67; 130.35)	
	BICF2P776685	G/G	16 (19.5%)	20 (55.6%)	1.00		
		A/G	39 (47.6%)	15 (41.7%)	0.13	(0.13; 0.75)	8.9×10^{-6}
		A/A	27 (32.9%)	1 (2.8%)	0.00	(0.00; 0.24)	
8	*TIGRP2P107898_rs9044787*	G/G	46 (56.8%)	6 (16.7%)	1.00		
		A/G	31 (38.3%)	13 (36.1%)	3.22	(1.10; 9.37)	5.7×10^{-8}
		A/A	4 (4.9%)	17 (47.2%)	32.58	(8.18; 129.78)	
14	*BICF2P797481*	G/G	19 (23.2%)	23 (63.9%)	1.00		
		A/G	39 (47.6%)	12 (33.3%)	0.25	(0.10; 0.62)	8.8×10^{-6}
		A/A	24 (29.3%)	1 (2.8%)	0.03	(0.00; 0.28)	
16	*BICF2P1314057*	G/G	60 (73.2%)	8 (23.5%)	1.00		
		A/G	20 (24.4%)	22 (64.7%)	8.25	(3.18; 21.43)	3.2×10^{-6}
		A/A	2 (2.4%)	4 (11.8%)	15.00	(2.36; 95.47)	
19	*TIGRP2P268994_rs8894778*	G/G	56 (68.3%)	10 (28.6%)	1.00		
		A/G	15 (18.3%)	23 (65.7%)	8.59	(3.37; 21.89)	4.8×10^{-6}
		A/A	11 (13.4%)	2 (5.7%)	1.02	(0.20; 5.30)	
23	*BICF2P373712*	A/A	21 (26.2%)	26 (72.2%)	1.00		
		G/A	29 (36.2%)	8 (22.2%)	0.22	(0.08; 0.59)	2.9×10^{-6}
		G/G	30 (37.5%)	2 (5.6%)	0.05	(0.01; 0.25)	
37	*BICF2G630136001*	G/G	42 (51.2%)	4 (11.4%)	1.00		
		A/G	23 (28.0%)	29 (82.9%)	13.24	(4.14; 42.34)	1.5×10^{-7}
		A/A	17 (20.7%)	2 (5.7%)	1.24	(0.21; 7.39)	

CHR – chromosome; OR – odds ratio; CI – confidence interval

Table 6. The risk of mammary gland tumours associated with particular genotypes within single nucleotide polymorphisms (SNPs) for the overdominant model

CHR	SNP	Genotype	Genotype number (percentage)		OR (95% CI)	P-value
			Control	Case		
1	*BICF2P343250*	G/G-A/A	43 (52.4%)	33 (91.7%)	0.10	9.99×10^{-6}
		A/G	39 (47.6%)	3 (8.3%)	(0.03; 0.35)	
8	*BICF2P158200*	G/G-A/A	71 (86.6%)	16 (44.4%)	8.07	3.03×10^{-6}
		A/G	11 (13.4%)	20 (55.6%)	(3.23; 20.13)	
	BICF2P441276	G/G-A/A	62 (75.6%)	10 (30.3%)	7.13	6.16×10^{-6}
		A/G	20 (24.4%)	23 (69.7%)	(2.91; 17.49)	
10	*BICF2S23760334*	G/G-A/A	65 (79.3%)	13 (36.1%)	6.76	6.52×10^{-6}
		A/G	17 (20.7%)	23 (63.9%)	(2.85; 16.06)	
11	*BICF2S23118240*	G/G-A/A	51 (62.2%)	35 (97.2%)	0.05	7.59×10^{-6}
		A/G	31 (37.8%)	1 (2.8%)	(0.01; 0.36)	
19	*TIGRP2P268994_rs8894778*	G/G-A/A	67 (81.7%)	12 (34.3%)	8.56	7.49×10^{-7}
		A/G	15 (18.3%)	23 (65.7%)	(3.50; 20.95)	
37	*BICF2G630129768*	A/A-G/G	57 (69.5%)	9 (25.0%)	6.84	5.72×10^{-6}
		G/A	25 (30.5%)	27 (75.0%)	(2.81; 16.64)	
	BICF2G630136001	G/G-A/A	59 (72.0%)	6 (17.1%)	12.4	2.14×10^{-8}
		A/G	23 (28.0%)	29 (82.9%)	(4.55; 33.78)	
X	*BICF2S23347259*	G/G-A/A	62 (75.6%)	11 (31.4%)	6.76	6.79×10^{-6}
		A/G	20 (24.4%)	24 (68.6%)	(2.82; 16.20)	

CHR – chromosome; OR – odds ratio; CI – confidence interval

Table 7. The risk of mammary gland tumours associated with particular genotypes within single nucleotide polymorphisms (SNPs) for the log-additive model

CHR	SNP	Genotype	OR	95% CI	P-value
1	*BICF2P825735*	0, 1, 2	4.66	(2.17; 9.99)	9.95×10^{-6}
	BICF2P501513	0, 1, 2	4.21	(2.11; 8.37)	5.17×10^{-6}
	BICF2P776685	0, 1, 2	0.23	(0.12; 0.46)	2.40×10^{-6}
	BICF2P345133	0, 1, 2	3.25	(1.87; 5.67)	8.34×10^{-6}
3	*BICF2G630704243*	0, 1, 2	0.23	(0.11; 0.47)	4.23×10^{-6}
	BICF2G630704438	0, 1, 2	0.28	(0.15; 0.52)	8.86×10^{-6}
8	*TIGRP2P107898_rs9044787*	0, 1, 2	5.46	(2.76; 10.81)	1.60×10^{-8}
13	*TIGRP2P176993_rs9197628*	0, 1, 2	5.39	(2.50; 11.61)	2.19×10^{-6}
14	*BICF2P797481*	0, 1, 2	0.22	(0.11; 0.45)	1.62×10^{-6}
	BICF2P720053	0, 1, 2	0.23	(0.12; 0.46)	2.89×10^{-6}
16	*BICF2P1314057*	0, 1, 2	5.87	(2.65; 13.03)	1.16×10^{-6}
23	*BICF2P373712*	0, 1, 2	0.23	(0.12; 0.45)	4.49×10^{-7}
24	*BICF2S23648284*	0, 1, 2	4.78	(2.27; 10.08)	6.34×10^{-6}
30	*BICF2G630397948*	0, 1, 2	3.39	(1.91; 6.03)	6.58×10^{-6}
34	*BICF2S23049081*	0, 1, 2	3.92	(2.07; 7.41)	2.84×10^{-6}
35	*BICF2P28560*	0, 1, 2	0.22	(0.10; 0.46)	2.81×10^{-6}
37	*BICF2G630127550*	0, 1, 2	0.26	(0.13; 0.51)	9.59×10^{-6}

CHR – chromosome; OR – odds ratio; CI – confidence interval

For the codominant model analysis, the reference genotype is a major allele homozygote. The appearance of each subsequent minor allele in the genotype increased the probability of developing a mammary gland tumour in the case of the *BICF2P448058*, *BICF2P501513*, *TIGRP2P107898_rs9044787* and *BICF2P1314057* markers (Table 5). A particularly noticeable increase in the likelihood of developing the disease was noted for the minor allele homozygous in the case of *TIGRP2P107898_rs9044787* (OR = 32.58). An inverse tendency, *i.e.* a decrease in the probability of developing the tumour with each additional minor allele in the genotype, was demonstrated for *BICF2P776685*, *BICF2P797481* and *BICF2P373712*. For *TIGRP2P268994_rs8894778* and *BICF2G630136001*, the heterozygous genotype indicated the highest risk.

The analysis based on the overdominant model made the identification possible of markers for which the heterozygous genotype was the risk factor of tumour incidence (Table 6). These included *BICF2P158200* (OR = 8.07), *BICF2P441276* (OR = 7.13), *BICF2S23760334* (OR = 6.76), *TIGRP2P268994_rs8894778* (OR = 8.56), *BICF2G630129768* (OR = 6.84), *BICF2G630136001* (OR = 12.4) and *BICF2S23347259* (OR = 6.76). The same conclusion regarding *TIGRP2P268994_rs8894778* and *BICF2G630136001* was drawn on the basis of the codominant model analysis. In the case of the remaining two markers (*BICF2P343250* and *BICF2S23118240*), the appearance of heterozygous genotypes decreased the likelihood of developing the disease.

Finally, the markings 0, 1, and 2 were observed to correspond to the number of minor alleles in each genotype in the log-additive model. In 9 of 17 SNPs (*BICF2P825735*, *BICF2P501513*, *BICF2P345133*, *TIGRP2P107898_rs9044787*, *TIGRP2P176993_rs9197628*, *BICF2P1314057*, *BICF2S23648284*, *BICF2G630397948* and *BICF2S23049081*), the appearance of minor alleles in the genotype increased the risk of developing mammary gland tumours (Table 7), with odds ratios ranging from 3.25 (*BICF2P345133*) to 5.87 (*BICF2P1314057*). The remaining 8 SNPs (*BICF2P776685*, *BICF2G630704243*, *BICF2G630704438*, *BICF2P797481*, *BICF2P720053*, *BICF2P373712*, *BICF2P28560* and *BICF2G630127550*) decreased the carrier's risk of developing the disease with each subsequent minor allele in the genotype. The odds ratios ranged from 0.22 (*BICF2P797481* and *BICF2P28560*) to 0.28 (*BICF2G630704438*). The results obtained using this model were consistent with those obtained using the codominant model (for BICF2P501513, *BICF2P776685*, *TIGRP2P107898_rs9044787*, *BICF2P797481*, *BICF2P1314057* and *BICF2P373712*), the dominant model (for *BICF2G630704243*, *TIGRP2P176993_rs9197628*, *BICF2P1314057* and *BICF2P373712*) and the recessive model (for *TIGRP2P107898_rs9044787* and *BICF2S23049081*).

Discussion

In our study, we identified 40 SNPs located on 22 chromosomes that showed a statistically significant relationship with the possibility of CMT. These results correspond with those obtained by Melin *et al.* (27), as two significant SNPs were found on chromosome 11 (*BICF2P576198* and *BICF2S23118240* for the recessive and overdominant model, respectively). However, they were located on different areas of chromosome 11 to those observed in previous studies. In addition, the results of our research did not show any significant SNPs related to CMT on chromosome 27. Encouragingly, Karlsson *et al.* (19) also found no statistically significant SNPs on chromosome 27 when conducting a GWAS search for SNPs associated with osteosarcoma in three dog breeds (greyhounds, Rottweilers and Irish wolfhounds), although they found one (*BICF2P133066*) on chromosome 11. These differences may result from the lower homogeneity of the research group in our study and the lower number of dogs diagnosed with CMT.

In the group of SNPs that were indicated as significant in our GWAS analysis, several transpired to be located within genes that may play an important role in mammary gland cancer. One of the important polymorphic sites found in *BICF2P825735* in our study is located on chromosome 1 at position 41988950 within the *ARMT1* gene. This gene is located upstream of the *ESR1* gene and previous research confirmed the co-regulation of both genes and their fusion during breast cancer or endometriosis (26, 39). It is worth noting that the results of some studies (6) indicated a significant relationship between genetic variability in the *ESR1* gene (*rs397512133*, *rs397510462*, *rs397510612*, *rs851327560*, *rs852887655*, *rs852684753* and *rs852398698*) and less aggressive clinical and pathological traits of CMT, a later onset of the disease, smaller tumour sizes, and hence better prognoses.

In addition, we found one SNP on chromosome 1 (*BICF2S2334324*) within the *ZCCHC2* gene, which corresponds to the results of Karlsson *et al.* (19), who also observed one SNP near this gene (*BICF2P1225396*) associated with the risk of osteosarcoma in Irish wolfhounds. The role of this gene during carcinogenesis appears ambiguous, as studies by Dai *et al.* (10) showed that the zinc finger protein ZCCHC2 inhibits the development of retinoblastoma by inhibiting HectH9-mediated K63-linked polyubiquitination and activation of c-Myc. Furthermore, studies by other authors have shown that this gene may affect immunity and disease resistance (41, 42).

On chromosome 30, where the *RAD51* gene is situated, there was an SNP (*BICF2G630397948*) in the *THSD4* gene. Together with *ERS1*, *TFF1* or *FOXA1* genes, among others, this gene demonstrates downregulation in breast cancer and a relationship with better survival prognoses (4). We also observed a significant polymorphism within the *RBPMS* gene on chromosome 16, where the *CDKN2A* gene is located. Studies showed that it played an important role in ovarian cancer by affecting cell proliferation and tumour sensitivity to therapy (34). Furthermore, the *ARHGEF19* gene located on chromosome 2 and the *LIMCH1* gene located on chromosome 13, within each of which we found one SNP (*TIGRP2P33447_rs8841788* and

TIGRP2P176993_rs9197628, respectively), were associated with an increased risk of breast cancer (3, 30).

Many of the SNPs that have been associated with canine mammary cancer are located in regions of the chromosomes where genes associated with carcinogenesis are also found. On chromosome 1, five out of seven SNPs are in the area between base pair 65,212,166 and base pair 67,922,585, where the *HEY2* gene is located, an essential element of the Notch signalling pathway (40). Chromosome 3 is characterised by SNPs located within/near the *Homer1*, *DMGDH* or *MSH3* genes, which are also important in the regulation and development of cancer (9, 24, 28). In the base pair 34,943,508 to base pair 569,645,583 area of chromosome 8, where three important SNPs were selected, there are numerous genes related to cancer, *i.e.* genes from the Six family (Six1, Six4 and Six6), *ESR2* or *MAX*. The fourth SNP (*TIGRP2P33447_rs8841788*) identified on chromosome 8 was in the *NPAS3* gene, a member of the neuronal PAS transcription factor gene family, which has diverse roles that include tumour development (8, 12). In the area between base pair 42,346,461 and base pair 42,358,113 on chromosome 15, three polymorphisms are located within/near the *ASCL1* gene (*TIGRP2P201649_rs8752112*, *BICF2P352914* and *BICF2S23334099*) which played an important role in the development of lung cancer (2). In turn, Karlsson *et al.* (19) showed one significant SNP (*TIGRP2P22000071*, position 38,987,072) in this area associated with the risk of osteosarcoma in Rottweilers. Two other SNPs were located on chromosome 35 in the region of base pair 15,603,710 to base pair 20,654,940. One of them (*BICF2P28560*) is located within the *ATXN1* gene, which enhanced E-cadherin expression at the protein and mRNA levels in MCF-7 breast cancer cells when over-expressed (21).

Although CMTs are among the most common tumours affecting bitches and seem to be quite extensively studied, the innate carcinogenic process is still under-researched, especially with regard to its genetic background. For this reason, any research aimed at identifying the genetic profile, especially regarding the impact of SNPs on the risk of mammary gland cancer in dogs, is extremely important. The results obtained in our GWAS analysis examining the occurrence of mammary gland cancer in dogs showed that the basis for the development of this tumour is highly complex. The SNPs indicated in our study are located in areas of genes related to the processes of carcinogenesis, tumour development, and metastasis, as well as determining the susceptibility to a particular treatment method. As our results are promising, it seems necessary to screen a larger number of individuals for the selected SNPs, as well as to examine the linkage disequilibrium within the selected regions of the genome.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: This research was supported by the Wrocław University of Environmental and Life Sciences (Poland) as part of the "Innovative Scientist" Ph.D. research programme, no. B030/0036/20. The APC was co-financed by Wrocław University of Environmental and Life Sciences.

Animal Rights Statement: In accordance with the relevant legislation, collecting material from bitches during the performance of veterinary services does not require the approval of the Ethics Committee.

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