Original Article

Investigation of *p27* tumor suppressor gene (*CDKN1B*) polymorphisms in dogs with malignant mammary tumors

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Abstract

Mammary tumors are the most common neoplasm in dogs, with a high mortality rate. The development of canine mammary tumors (CMT) is multifactorial, but different incidence rates between breeds suggest the effects of genetic risk factors. Many CMT-associated candidate genes were reported, mainly involved in cell cycle control. This study aims to investigate *p27* tumor suppressor gene polymorphisms in dogs with mammary tumors and analyze the association between variations and CMTs. For this purpose, case and control groups were formed from 22 dogs diagnosed with malignant mammary tumors and 10 dogs with healthy mammary glands. The whole canine *p27* gene was amplified and sequenced, including three exons, introns, and UTRs. Seven SNPs were identified in the 3'UTR of the *p27* gene. The detected SNPs were as follows: A/C transversion at position 27:33916126, A/G transition at position 27:33915987, A/G transition at position 27:33915861, A/G transition at position 27:33915847, A/G transition at position 27:33915797, A/G transition at position 27:33915713, A/C transversion at position 27:33915684. No significant association was observed between these SNPs and canine mammary tumors. The coding region of the *p27* gene of the dogs in this study was highly conserved and monomorphic. Further research on *p27* polymorphisms and gene expression and their effects on mammary tumor development would shed light on the molecular basis of mammary cancers in dogs.

Keywords: *p27*, canine mammary tumor, dog, SNP, *CDKN1B*

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Introduction

Canine mammary tumors (CMT) are the most common neoplasm in female dogs, representing 50% of all tumors. CMTs are either the primary tumors of the mammary gland or metastatic tumors from other organs and tissues (Thejaswini *et al*., 2022). The development of the CMTs is multifactorial, but different incidence rates between dog breeds support the effect of genetic risk factors. Many CMT-associated candidate genes have been reported, mainly involved in cell cycle control, DNA damage recognition, and repair pathways (Borge *et al*., 2011).

CMTs exhibit many clinical and molecular similarities to human breast cancer (Bird *et al*., 2011; Gray *et al*., 2020). Hence, dogs and humans share common genes associated with mammary cancer risk, such as *BRCA1, BRCA2, TP53, PTEN, CHEK2, TOX3, ERBB2, BRIP1, and STK11* (Enginler *et al*., 2014). One of the genes associated with mammary cancer is *CDKN1B* (Also referred to as the *p27* gene in this article). This gene encodes for the CDK inhibitor $p27^{kip1}$ protein (*p27*), a cyclin-dependent kinase (CDK) inhibitor. *p27* participates in biological processes like cell proliferation, differentiation, migration, and apoptosis (Polyak, 2006). *p27* inhibits cell cycle progression through binding cyclin-CDK complexes (Kumar *et al*., 2015; Zou and Lin, 2021). The *p27* protein inhibits the activity of CDK2–cyclin E and CDK4–cyclin D complexes at the G1-phase and acts as a CDK inhibitor that prevents the S-phase transition in the cell cycle (James *et al*., 2008). In addition, other physiological factors regulating cell proliferation, such as contact inhibition and cAMP, act through this protein (Sherr and Roberts, 1995; Slingerland and Pagano, 2000). Therefore, the *p27* is accepted as an effective tumor suppressor. However, studies in the last decade have revealed an oncogenic activity due to its cytoplasmic localization. Cytoplasmic *p27* functions in processes associated with tumor development and progression (Currier *et al*., 2019).

The *CDKN1B* gene is mutated in some human cancer subtypes, including luminal breast cancer, prostate cancer, and small intestine neuroendocrine tumors (Cusan *et al*., 2018). Apart from somatic mutations, germline *CDKN1B* risk variants have been described in hereditary tumors, such as multiple endocrine neoplasia (MEN)-like syndromes (Lee *et al*., 2013)and familial prostate cancer in humans (Chang *et al*., 2004). This study aims to determine germline variants in the canine *CDKN1B* gene and investigate their association with CMTs in dogs.

Materials and Methods

Animals: This study's case and control groups consisted of female dogs that had visited Istanbul University-Cerrahpasa, Faculty of Veterinary Medicine, Department of Obstetrics and Gynaecology in Turkiye. This study was approved by the Istanbul University Animal Experiments Local Ethics Committee decision dated 02/03/2018 and numbered 2018/08. Of 27 dogs with malignant tumors, five were diagnosed with sarcoma and 22 with carcinoma. Therefore, this study continued with 22 dogs diagnosed with carcinoma. Data regarding breed, age, tumor location, class, and ovariohysterectomy status of dogs were recorded (Tables 1 and 2).

Sample Collection: The diagnosis of mammary tumors was made in line with the anamnesis, physical examination, and histopathological diagnosis. Tissue samples were obtained by surgical biopsy and were sent to the Department of Pathology for histopathological evaluation. Histopathological diagnosis was evaluated on hematoxylin and eosin-stained sections by the World Health Organization's (WHO) classification for canine mammary tumors (WHO, 1999). Before the operations, 5 ml blood was taken from the vena cephalica antebrachial into the vacuumed tubes containing EDTA.

Table 1 Control number, breed, age, body weight, ovariohysterectomy (OVH) information of female dogs included in the control group.

Control no.	Breed	Age	Body Weight	OVH
Control 1	Mixed	12	22 kg	-
Control 2	Mixed	10	18 kg	-
Control 3	Collie	11	18 kg	-
Control 4	Mixed	13	32 kg	-
Control 5	Kangal	10	28 kg	-
Control 6	Akbaş	10	35 kg	-
Control 7	Kangal	14	38 kg	-
Control 8	German Shepherd	10	23 kg	-
Control 9	German Shepherd	9	23 kg	-
Control 10	Mixed	11	17 kg	-

Table 2 Case number, breed, age, tumor localization (TL), ovariohysterectomy status (OVH), tumor class, and tumor grade information of dogs included in the case group.

Case no.	Breed	Age	OVH (Ovariohysterectomy)	Tumor Localization	Tumor Class	Tumor Grade
1	Terrier	15	-	Left cranioabdominal	Adenocarcinoma	Grade3
				Left caudoabdominal	Adenocarcinoma	Grade3
2	Yorkshire Terrier	11	-	Left thoracic cranial	Tubular adenocarcinoma	Grade2
				Left-right cranioabdominal	Complex adenocarcinoma	Grade2
				Left inguinal	Complex adenocarcinoma	Grade2
3	German Shepherd	10	-	Left craniothoracic	Tubular adenocarcinoma	Grade1
				Left inguinal	Squamous cell carcinoma	-
				Right caudothoracic	Carcinoma -mixed type	Grade1
4	German Shepherd	7	-	Left cranioabdominal	Anaplastic adenocarcinoma	Grade3
				Right caudothoracic	Tubular adenocarcinoma	Grade1
5	Mixed	13	-	Left cranioabdominal	Tubulopapillary adenocarcinoma	Grade2
				Left caudoabdominal	Tubulopapillary adenocarcinoma	Grade1
6	American Cocker Spaniel	8	+	Right cranioabdominal	Adenocarcinoma	Grade3
7	Terrier	8	-	All lobes	Tubular adenocarcinoma	Grade1
8	Pekingese	5,5	-	Left caudoabdominal	Complex adenocarcinoma	Grade2
9	German Shepherd	9	-	Right caudoabdominal	Complex adenocarcinoma	Grade1
10	Mixed	13	-	Left caudoabdominal	Complex adenocarcinoma	Grade2
				Right inguinal	Complex adenocarcinoma	Grade2
11	Pinscher	16	-	Left caudoabdominal	Complex adenocarcinoma	Grade2
				Left cranioabdominal	Complex adenocarcinoma	Grade2
				Right inguinal	Simple adenocarcinoma	Grade3
12	Terrier	13	+	Right craniothoracal	Carcinosarcoma	Grade2
				Right cranioabdominal	Complex adenocarcinoma	Grade2
				Right caudoabdominal	Complex adenocarcinoma	Grade2
13	Kangal	5	-	Right inguinal	Adenocarcinoma	Grade3
14	Golden Retriever	10	-	All lobes	Papillary adenocarcinoma	Grade3
15	Terrier	9	+	Left inguinal	Simple adenocarcinoma	Grade1
16	Mixed	13	-	Right inguinal	Carcinoma in situ	Grade2
				Left inguinal	Tubulopapillary adenocarcinoma	Grade2
17	Poodle	13	-	Left-right craniothoracal	Tubular adenocarcinoma	Grade2
				Left-right caudothoracic	Tubular adenocarcinoma	Grade2
				Left-right cranioabdominal	Tubular adenocarcinoma	Grade2
				Left-right caudoabdominal	Tubular adenocarcinoma	Grade2
				Right inguinal	Tubular adenocarcinoma	Grade2
				Left inguinal	Complex adenocarcinoma	Grade2

OVH (+): neutrized, OVH(-): non-neutrized

Table 3 Primers used in the sequencing of the *p27* gene.

Primer No.	Primer Sequence	Tm (°C)
*P27*_1F	5′ TGTTTTCCGAGAGAGGGAGA 3′	57
*P27*_1R	5′ GAAAAGCAAGCTGCGGTTAG 3′	57
*P27*_2F	5′ TTAAAGGCCACCGGGAATGA 3′	57
*P27*_2R	5′ TTGGGTATCTCGGGGTGTGA 3′	59
*P27*_3F	5′ TCCATTGCTCAGGTATTTCACAAC 3′	59
*P27*_3R	5′ ACTGCTTCTCTCCATGCAAGT 3′	58
*P27*_4F	5′ GAGGGTGGGGCTGAGGA 3′	60
*P27*_4R	5′ GTGTCTACATAGCCCAAAGTCCA 3′	61
*P27*_5F	5′ ATTCTGGGCAAAATTCTGGGGTA 3′	59
*P27*_5R	5′ GCAACCTTTTAAGCATAGCCATAT 3′	58

DNA Extraction, PCR, and Sequencing: Genomic DNA was isolated from whole blood samples with a Roche High Pure PCR Template Preparation Kit. Eight regions, consisting of 5287 bp, were targeted for the amplification of the canine *p27* gene (Accession number: NC_051831.1). The primers were designed according to the nucleotide sequence taken from the Ensembl database (https://www.ensembl.org/index.html). The sequences of the primers and their annealing temperatures (Tm) are given in Table 3.

PCR amplifications were carried out in a reaction volume of 25 µl using 10XPCR buffer (100 mM KCl, 20 mM Tris HCl (pH 8.0), 0.1 mM EDTA, 0.5 mM PMSF, 1 mM DTT, 50 % glycerol) and 4.5 mM $MgCl_2$, 10 pmol of each primer, 100µM dNTP, 1 U Taq polymerase, 50–100 ng genomic DNA and dH_2O.

Touch-down PCR was performed with conditions as follows: initial denaturation at 95°C for three minutes followed by 15 cycles of denaturation at 95°C for 20 seconds, annealing at 60°C for 25 seconds (by decreasing 0.5°C in each cycle), elongation at 72°C for 50 seconds. Then, 19 cycles of denaturation at 95°C for 15 seconds, annealing at 52°C for 20 seconds, elongation at 72°C for 40 seconds, and final extension at 72°C for five minutes. PCR products were run through 2% agarose gel to check the amplification results.

Identifying Sequence Variations: The obtained DNA sequences of the *p27* gene were aligned using the Clustal W program in the MEGA 11 software program (Tamura *et al.*, 2021). The samples included in the case and control groups were compared with each other and the reference sequence of the Canis familiaris *p27* gene (GenBank Accession number: NC_051831.1) to detect polymorphisms.

Statistical Analysis: Samples with malignant mammary tumors and the control group were compared regarding allele and genotype frequencies of SNPs detected in the *p27* gene. Allele frequencies and genotype frequencies were calculated using the SPSS 25.0 software program. The statistical significance of the relationship between SNPs and cases was determined using the Pearson bilateral $\chi 2$ (chi-square) test in the SPSS 25.0 program (IBM, 2017). Unconditional logistic regression analysis determined odds ratios (with a 95% confidence interval).

Logistic regression analysis was used to determine the factors affecting tumor formation. In the model used for this analysis, breed groups (large breed, small breed and mixed breed), ovariohysterectomy (OVH) status and SNPs (27:33916126, 27:33915987, 27:33915861, 27:33915847, 27:3391579, 27:33915713, 27:33915684) were determined as categorical variables, age and weight were added to the model as covariates. 27:33915987 and 27:33915847 regions were excluded from the analysis because the prediction percentage decreased when the regions were added to the regression model. The breeds were grouped based on the wither's height (> 50 cm large breed, < 50 cm medium and small breed) in the FCI breed nomenclature system (FCI, 2013; Pastor *et al.*, 2018). The statistical significance level was determined as $p < 0.05$.

Results

Many polymorphisms have been reported for the canine *p27* gene so far. Our study determined seven novel SNPs, including 5 A/G transitions and 2 A/C transversions, in the 3'UTR region of the canine *p27* gene. The previously reported polymorphisms in the databases were not detected in this study. It has been noted that the coding region was highly conserved in our study group. No polymorphism was observed except those in 3′UTR.

Minor allele frequencies of the SNPs and their relationship with CMTs are given in Table 4. No significant association was observed between seven SNPs and CMTs. The minor allele frequencies of the SNPs at positions 27:33916126 and 27:33915987 in all the samples were calculated as 0.10 and 0.11, respectively. The minor alleles of the other five SNPs varied from 0.43 to 0.45.

Genotype frequencies are given in Table 5. All the SNPs except A/C polymorphism at position 27:33915684, had two genotypes: homozygous genotype for major allele and heterozygous genotype. Three genotypes were observed for the A/C polymorphism at position 27:33915684. The results showed that there was a high heterozygosity in SNPs at positions 27:33915861, 27:33915847, 27:33915797 and 27:33915713, with the frequencies 85.7%, 90.5%, 87.5% and 87.5%, respectively. No statistically significant association was determined between the genotypes and CMTs.

The logistic regression analysis revealed that age, weight, and breed group did not significantly affect mammary tumor formation in dogs. A co-analysis of the various factors and the SNPs' genotypes also confirmed no significant relationship between the SNPs and CMTs (Table 6).

Table 4 Allele frequencies of SNPs in the *p27* gene and their relationship with CMT

SNP	Genome Position	Alleles	Minor allele	Minor allele frequency		Odds ratios (95% confidence interval)	χ^2
				All samples	Cases		
Novel SNP	27:33916126	A/C	C	0.10	0.11	0.520 (0.56-4.827)	0.341 Ns
Novel SNP	27:33915987	A/G	G	0.11	0.11	0.918 (0.162-5.207)	0.009 Ns
Novel SNP	27:33915861	A/G	A	0.43	0.43	0.938 (0.308-2.852)	0.013 Ns
Novel SNP	27:33915847	A/G	A	0.45	0.45	1.010 (0.346-2.944)	0.000 Ns
Novel SNP	27:33915797	A/G	A	0.43	0.43	0.938 (0.308-2.852)	0.013 Ns
Novel SNP	27:33915713	A/G	A	0.43	0.43	0.938 (0.308-2.852)	0.013 Ns
Novel SNP	27:33915684	A/C	A	0.45	0.45	1.033 (0.340-3.135)	0.003 Ns

Ns: Non-significant, *$p < 0.05$

Table 5 Genotype frequencies of SNPs in the *p27* gene and their relationship with CMT

Genome Position	Genotype Frequency (%)																				
	27:33916135			27:33915987			27:33915861			27:33915847			27:33915797			27:33915713			27:33915684			
Group	AA	AC	χ^2	AA	AG	χ^2	GG	AG	χ^2	GG	AG	χ^2	GG	AG	χ^2	GG	AG	χ^2	AA	AC	CC	χ^2
Case	77.3	22.7	0.384	77.3	22.7	0.030	14.3	85.7	0.055	9.5	90.5	0.055	14.3	87.5	0.055	14.3	87.5	0.055	28.6	33.3	38.1	0.346
			Ns			Ns			Ns			Ns			Ns			Ns				Ns
Control	87.5	12.5		80	20		13.3	86.7		9.7	90.3		11.1	88.9		11.1	88.9		22.2	44.4	33.3	

Ns: Non-significant, *$p < 0.05$

Table 6 The effects of various factors on tumor formation

Factors	B	S.E.	Sig.	Exp(B)
Age	-0.883	.646	0.172	0.413
Weight	-0.132	.103	0.197	0.876
27:33916126 (AC)				
(Ref.)				
27:33916126 (AA)	-1.873	1.901	0.325	0.154
27:33915861 (GG)				
(Ref.)				
27:33915861 (AG)	20.146	30463.894	0.999	561234486.547
27:33915797 (GG)				
(Ref.)				
27:33915797 (AG)	-18.978	25110.720	0.999	0.000
27:33915713 (GG)				
(Ref.)				
27:33915713 (AG)	18.966	37524.915	1.000	172486544.028
27:33915684 (CC)			0.394	
(Ref.)				
27:33915684 (AA)	-1.359	2.577	0.598	0.257
27:33915684 (AC)	-3.311	2.427	0.173	0.036
Breed group 3 (mixed)			0.984	
(Ref.)				
Breed group 1 (small-medium)	40.035	17248.208	0.998	243733607530081408.000
Breed group 2 (large)	-0.454	2.520	0.857	0.635
OVH (-)				
(Ref.)				
OVH (+)	4.275	51285.611	1.000	71.849
Constant	-8.657	55512.569	1.000	0.000

Ref.: Reference value, OVH (+): neutrized, OVH(-): non-neutrized

While performing the logistic regression analysis, the subgroups specified in table (breed group 3 (mixed), for the 27:33915684 genome position CC, OVH (non-neutered), for the 27:33915861 genome position GG, for the 27:33915797 genome position GG, for the 27:33915713 genome position GG, for the 27:33916126 genome position AC) were selected for each variable as a reference.

Discussion

Compared to the mutations occurring in tumors, CMT-associated germline mutations and their role in tumorigenesis have been little studied and have remained under-researched. Because of the pivotal role of *p27* in tumor development, malignant progression, and metastasis (Klopfleisch and Gruber, 2009; Klopfleisch *et al*., 2010), germline variants in *CDKN1B* gene and their association with susceptibility to CMTs were investigated in this study. We identified seven novel SNPs in the 3′ UTR of the *CDKN1B* gene.

In humans, there are two major inherited cancer diseases, namely MEN-4 (Multiple Endocrine Neoplasia) Syndrome and Familial Prostate Cancer, which are associated with various germline mutations in the *CDKN1B* gene (Cusan *et al*., 2018). In the MEN-4 syndrome, missense mutations in the *CDKN1B* gene have been detected. In our study, no similar missense mutations were found in the case and control groups. Germline mutations that alter *p27* expression by causing changes in UTRs and are suggested to be related to MEN-1 and MEN-4 have also been identified. In a study focusing on familial prostate cancer, 10 germline variants were identified in the *CDKN1B* gene, including in the promoter region, exons, and introns (Chang *et al*., 2004).

In this study, statistical analysis of the discovered SNPs and CMTs did not reveal any significant association. Studies on humans indicate that the *p27* gene is associated with different types of cancer, but there are also controversial findings (Kawamata *et al*., 1995; Ferrando *et al*., 1996; Schöndorf *et al*., 2004; Dreijerink *et al*., 2006; Landa *et al*., 2010). In a previous study on the association between *CDKN1B* gene variants and breast cancer risk in 2359 female *BRCA1* and *BRCA2* mutation carriers, researchers reported that *CDKN1B* polymorphisms do not modify breast cancer risk among *BRCA1* or *BRCA2* carriers (Spurdle *et al*., 2009).

In this study, no polymorphism was detected in the coding region of the canine *CDKN1B* gene. The gene's coding region appears to be monomorphic in dog samples included in this study. It has been reported that the *CDKN1B* gene in humans also has a low diversity in the coding region. The only variant that could result in an amino acid change is the V109G polymorphism (Zhu *et al*., 2019). The V109G polymorphism of the human *CDKN1B* gene has been found in 11-26% of cancer patients, as well as in approximately 39% of healthy individuals and non-malignant cells of cancer tissue samples (Kawamata *et al*., 1995; Ferrando *et al*., 1996; Schöndorf *et al*., 2004). According to the studies on V109G polymorphism, it may increase susceptibility to breast cancer by reducing *p27* production (Wang *et al*., 2007), and it is associated with nodal involvement of tumor cells (Schöndorf *et al*., 2004). On the contrary, there are studies reporting no association between V109G variation and breast cancer risk in women (Onay *et al*., 2006; Ma *et al*., 2006). Figueiredo *et al*. (2007) found that this polymorphism is associated with elevated T-stage cancer and nodal involvement but not with breast cancer. No significant relationship was found between the seven SNPs detected in our study and breast cancer susceptibility in dogs. The possible effects of these SNPs on tumor characteristics, such as nodal involvement, T-stage, and survival in dogs, should be investigated further.

We observed a monomorphic character in the coding region of canine *CDKN1B*. No association between the novel SNPs in the 3′ UTR and CMTs was found. These results are consistent with the approach that posttranslational modifications may also be responsible for the impact of *p27* on tumor development. Modifications, such as phosphorylation, ubiquitination, and sumoylation, are thought to alter the action of *p27* and its interaction with other proteins (Cusan *et al*., 2018). *p27* is an "intrinsically disordered" protein (IDP). IDPs are proteins with disordered regions and do not acquire an ordered structure without interacting with other macromolecules. Therefore, post-translational modifications significantly impact functional regulation (Dyson and Wright, 2005; Cusan *et al*., 2018). The post-translational modifications might explain the absence of a CMT-associated polymorphism in the CDKN1B gene in this study.

All SNPs detected in our study are in the 3'UTR region of the gene. Variants in the UTRs do not alter protein sequence but may have crucial functions in regulating gene expression (Steri *et al*., 2018). The 3′UTR is located downstream of the coding sequence and is involved in regulatory mechanisms, such as RNA stability, mRNA translation, and localization. This region has binding sites for miRNAs. Hence, any variation in the sequence of the 3′UTR can alter or inhibit the binding of miRNAs, resulting in alterations in gene expression. (Gramantieri *et al*., 2009). Although our study could not detect a statistically significant relations, these novel SNPs may be effective in the *p27* expression. The total number of individuals in this study's case and control groups may not be sufficient to reveal the possible impact. It can be recommended that further studies be conducted on more patients regarding these SNPs, which were identified for the first time in dogs.

A study on dogs with mammary tumors reported that miR-29b and miR-21 were statistically significant in cancer samples (Boggs *et al*., 2008). Various studies have also reported that these two miRNAs, which are significant in canine mammary tumors, are associated with the *p27* protein. Overexpression of *miR-29b* has been noted to significantly increase the percentage of cells in the G1 phase by inducing expression of the cell cycle-dependent kinase inhibitors p21 and *p27* (Amodio *et al*., 2012; Li *et al*., 2013). In another study investigating the impacts of miR-29b on porcine granulosa cells, it has been stated that the degradation of miR-29b reduces the expression of the *CDKN1B* gene (Hilker, 2021). Overexpression of miR-21 as an oncogene is associated with worse tumor differentiation, lymph node metastasis, and T stage (Sha *et al*., 2015). Another study on rats showed that miR-21 indirectly leads to the inhibition of p21/*p27* expression (Li *et al*., 2014). We might suggest that the relationship between these miRNAs and the polymorphisms observed in this study should be investigated further.

In conclusion, seven SNPs were identified in the 3′ UTR of the *CDKN1B* gene, which was found not to be correlated with CMTs in dogs. Moreover, the coding region was highly conserved and harbored no variations in any samples. The interaction between *CDKN1B* mutations and *p27* protein expression, especially in dogs, has not yet been fully clarified. Further analysis of gene, mRNA, and protein levels will shed light on the relationship between the CDKN1B gene, its product *p27* protein, and CMTs.

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