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ORIGINAL ARTICLE

Detection of Clinically Relevant Mutated Genes in Canine Breast Cancer Using DNA Sequencing Data

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Abstract

Breast cancer is one of the most important canine cancers and is considered an ideal animal model for its human counterpart. Compared to human breast cancer, the clinically relevant mutated genes are less well-known. In this study, we sought to find clinically relevant mutated genes using DNA sequencing data through an in-silico analysis. Using a canine mammary tumor DNA sequencing dataset containing 183 samples, namely SRP159481, we first identified frequently mutated genes as those with various types of somatic mutations present in at least 5% of the studied population. Then, we evaluated the association between these frequently mutated genes and different clinicopathological features, including lymph node metastasis (LN+/LN-), estrogen receptor status (ER+/ER-), degree of malignancy (benign/malignant), and histopathological tumor type. We found 26 frequently mutated genes, which were reduced to 18 after removing the silent mutations. Among these genes, TP53 was found to be the most clinically relevant gene, because it had a significant relationship with all clinical features. The frequency of TP53 was significantly higher in malignant tumors compared to benign tumors, ER- tumors compared to ER+ tumors, and LN+ cases compared to LN- cases ($p<0.05$). In addition, the survival time in dogs with TP53 mutation was significantly shorter than in dogs without this mutation ($p<0.05$). Moreover, the frequency of mutations in PIK3CA and SLC6A8 was significantly higher in benign cases than in malignant cases ($p<0.05$). We also validated our findings regarding TP53 using an external dataset.

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Introduction

Breast cancer is a main neoplasm in dogs and is special in many ways. The estimated annual incidence for this tumor is 192 cases per 100,000 female dogs. It is estimated that 41-53% of canine breast cancers are pathologically malignant (1). It is one of the most important and common tumors in domestic dogs. Furthermore, canine breast cancer has been recognized as a valuable animal model for the human counterpart, as there are many comparable and similar biological and clinical features between the two species. Similar characteristics, such as pathological classification, molecular properties, biological behaviors, and response to treatment, have been reported by different studies (2-5). For example, there is considerable overlap between the gene expression profiles of human and canine breast cancer. Furthermore, genes involved in cell cycle and mitosis have been identified as important gene signatures for stratifying high-risk patients and predicting clinical outcomes and survival in humans and dogs (2, 6).

Extensive and comprehensive advanced omics analyses, such as microarray, DNA sequencing, and RNA sequencing, have been performed in human breast cancer. Therefore, the molecular machinery of human breast cancer has been investigated and identified in more detail at different levels, including epigenomics, transcriptomics, and genomics (7-10). However, there are relatively few studies on canine mammary tumors, and hence, limited information is available on their molecular aspects (11). One of the interesting areas of molecular analysis is finding clinically relevant mutations and chromosomal variations at the genomic level using DNA sequencing data. Using DNA sequence analysis in humans, various mutations have been reported that have a significant relationship with different clinical characteristics such as tumor type, incidence of metastasis, and degree of malignancy. For example, high mutation rates in TP53, ERBB2, PIK3CA, BRCA1, BRCA2, MSH1, PMS2, and ATM have been indicated in human breast cancer (12-15). Moreover, mutations in TP32, BRCA1, and BRCA2 in canine breast cancer were also investigated (16,17). Furthermore, a significant association between PIK3CA mutations and reduced survival was detected in ER-positive breast cancer (18). In addition, a study by Stucci et al. revealed that ATM gene mutations are positively correlated with a higher risk of ER-positive breast cancer, high-grade tumor, and lymph node metastasis (12). Various molecular techniques, including trench sequencing, next-generation sequencing (NGS), and third-generation sequencing (TGS), are used to detect different types of mutations in normal and cancerous tissues. Compared to traditional PCR methods, these techniques have high throughput, meaning they can examine many mutations in a tumor sample simultaneously (19). Based on this, we conducted a study using DNA sequencing data to find clinically relevant genes with different somatic mutations in canine mammary tumors. First, we identified frequently mutated genes and then examined their relationship with clinical features such as malignancy, metastasis, and survival time.

Materials and Methods

Collecting and Processing DNA Sequencing Data

Three major sequencing databases, including GEO (Gene Expression Omnibus), SRA (Sequence Read Archive), and ArrayExpress, were explored for datasets containing canine breast cancer.

DNA sequencing data. Our goal was to find datasets that had a reasonable number of samples (at least 20 samples) and complete clinical information. We found only two datasets, one with 183 and the other with 12 samples. Consequently, only one dataset was considered suitable. The larger dataset, DNA sequencing data in the SRA database with accession numbers PRJNA489159 and SRP159481, contained tumor and normal tissue information from 183 canine mammary tumor specimens and was used as the main dataset for subsequent analyses. These tumor samples were subjected to whole-exome sequencing in paired mode using Illumina HiSeq 2500 technology. In this dataset, the human blood genome was considered as normal tissue. The second dataset with GEO accession number GSE54535 contained only 12 samples with incomplete clinical data. No sequence analysis was performed in this dataset, but chromosomal aberrations were detected using the array-CGH technique. Hence, this dataset could not be used as sequencing data. However, we used it as a confirmatory dataset. See further for more details.

For the SRP159481 dataset, the data (reads) were downloaded in raw format (FASTQ format), and their quality was evaluated with the help of FASTQC software (20). In this analysis, the accuracy and quality of 5% of the total volume of data were checked as a representative, and if the quality was confirmed, the rest of the data was downloaded. In addition, if there were adapters, they were removed using Trimmomatic software (21). Then, we implemented aligning the raw reads against the dog genome (CanFam3.1) using the BWA tool (22) and the BWA-MEM algorithm. The output files of the previous step in SAM format were changed to BAM format using SAMtools (23). Alignments were subsequently calibrated by GATK (24), and duplicates were detected by Picard (Broad Institute).

Approximately, for all software, we tried to use the default parameters in our analysis.

Identification of Somatic Point Mutations and Insertions/Deletions (Indels)

The MuTect tool (25) was used to identify somatic gene mutations. In this analysis, the minimum coverage was 10X, and the mutation allele fraction was at least 10%. These mutations were annotated with the ANNOVAR tool (26). Also, the indels were identified through comparison of BAM files of tumor and normal tissue using the VarScan2 tool (27).

Identification of Frequently Mutated Genes and Their Gene Ontology (GO)

Genes that contain different types of somatic mutations in at least 5% of the studied population were considered as frequently mutated genes and were included in subsequent analyses. Also, we investigated the gene ontology of these important genes to identify their role in normal/abnormal biological pathways. GO analysis was performed by entering the final genes into the DAVID database (28).

Determining the Association between Frequently Mutated Genes and Clinicopathologic Characteristics

To determine the correlation between the frequently mutated genes and various clinicopathological features, Pearson's chi-square test was used at the level of 0.05. All clinical and non-clinical metadata of 183 studied cases are available in the GEO database. In this study, the evaluated characteristics included the presence or spread of metastasis to lymphatic vessels (LN+/LN-), estrogen receptor status (ER+/ER-), degree of malignancy (benign/malignant), and histopathological type of tumor. The types of tumors in terms of pathology included simple adenoma, simple carcinoma, complex adenoma, complex carcinoma, carcinoma in benign mixed tumor, osteosarcoma, fibrosarcoma, benign mixed tumor, and spindle cell carcinoma (malignant myoepithelioma). These analyses were performed using the SPSS 16 package at the $P < 0.05$ level.

Identification of Frequently Mutated Genes Associated with Survival

The association between survival time and frequently mutated genes was done using univariate Cox proportional hazard analysis. The survival analysis was studied using SPSS 16 software at $p < 0.05$.

Confirming the Presence of Chromosomal Aberrations in the Frequently Mutated Gene(s) Using an External Validation Dataset (GSE54535)

Although we did not find a suitable DNA sequencing dataset to serve as an external validation dataset, as a confirmatory step, we confirmed the presence of chromosomal aberrations (copy number variations (CNVs)) in the most frequently mutated gene(s) using a small dataset (GSE54535) containing 12 canine breast cancer samples. In this dataset, chromosomal aberrations were measured using the array-CGH method. The process of using these data to identify CNVs has been detailed elsewhere (29). Briefly, the log2 ratios (tumor/normal) of 389,318 array-CGH probes were downloaded and then averaged. Using the CGH-Explorer 3.2 tool (30), copy number variations were then detected through copy error analysis, with a false discovery rate (FDR) of 0.0001. After identifying CNVs, the association between CNVs of frequently mutated genes and the only available clinical metadata available in the dataset (ER status) was tested with the Pearson's chi-square test at the 0.05 level.

Results

Detection of the Frequently Mutated Genes

Our analysis showed that 26 genes listed in Table 1 had a frequency of at least 5% in our population. Different types of mutations included synonymous (silent), nonsense, missense, splicing, in-frame indel, and frameshift. Due to the lower significance of synonymous (silent) mutations, these mutations were not considered for subsequent analyses. For this reason, the number of important genes was reduced to 18, which included ANKRD17, PIK3CA, PTEN, KRAS, TP53, FSIP2, PCLO, SETD1A, TTN, PIK3R1, CELSR2, ND5, ATP7B, C25H2orf72, cOR51C4, FAM189A2, NKX1-2, and SLC6A8 (Figure 1 and Table 1). The excluded genes contained only silent mutations.

Gene Ontology of the Frequently Mutated Genes

The results of the GO analysis on the frequently mutated genes are summarized in Table 2. The results showed that many of these genes play important roles in the occurrence of various cancers, such as melanoma, glioma, lung cancer,

molecular pathways include PTEN-dependent cell cycle arrest and apoptosis, neurotrophin signaling pathway, ERBB2 signaling pathway, mTOR signaling pathway,

VEGF signaling pathway, PI3K-Akt signaling pathway, and ErbB signaling pathway.

Table 1. List of frequently mutated genes in canine mammary tumors

With silent mutation			Without silent mutation		
	Frequency	%		Frequency	%
ANKRD17	11	0.06	**ANKRD17**	11	0.06
ARFGEF3	10	0.05	**ATP7B**	10	0.05
ATP7B	10	0.05	**C25H2orf72**	11	0.06
C25H2orf72	11	0.06	**CELSR2**	14	0.08
CELSR2	16	0.09	**cOR51C4**	10	0.05
cOR51C4	10	0.05	**FAM189A2**	10	0.05
CSMD1	10	0.05	**FSIP2**	11	0.06
FAM189A2	10	0.05	**KRAS**	19	0.10
FSIP2	17	0.09	**ND5**	10	0.05
KRAS	19	0.10	**NKX1-2**	13	0.07
LY6K	11	0.06	**PCLO**	10	0.05
MKI67	11	0.06	**PIK3CA**	77	0.42
MT-CYB	11	0.06	**PIK3R1**	11	0.06
MT-ND1	16	0.09	**PTEN**	13	0.07
MYH6	12	0.07	**SETD1A**	13	0.07
ND5	12	0.07	**SLC6A8**	10	0.05
NKX1-2	14	0.08	**TP53**	16	0.09
PCLO	12	0.07	**TTN**	29	0.16
PIK3CA	77	0.42			
PIK3R1	11	0.06			
PTEN	13	0.07			
SETD1A	13	0.07			
SFXN4	16	0.09			
SLC6A8	10	0.05			
TP53	16	0.09			
TTN	29	0.16			

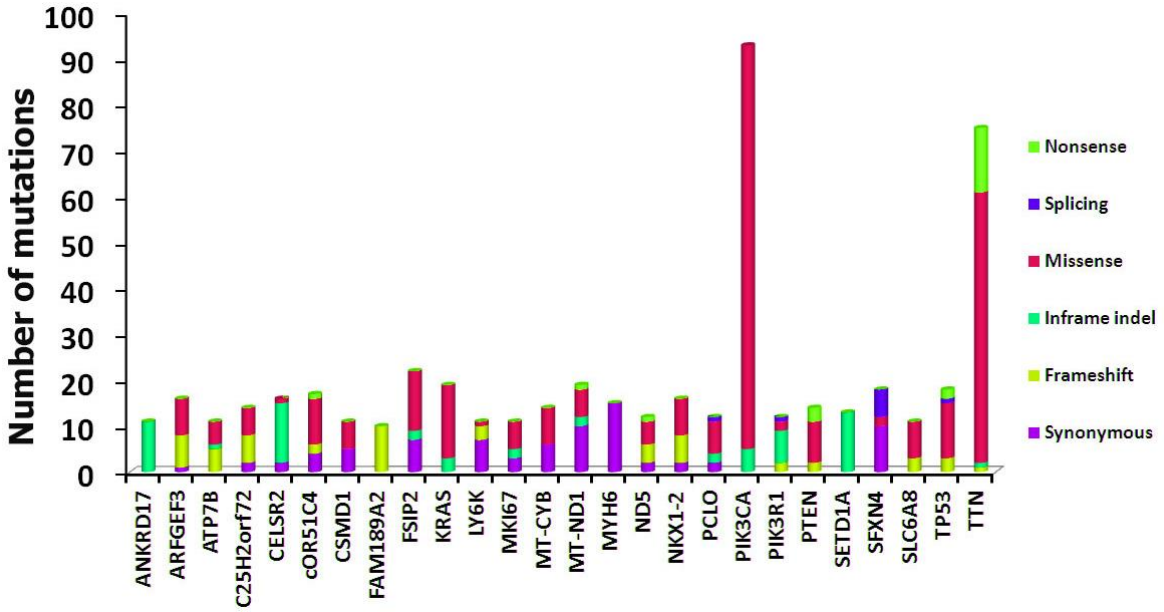

Figure 1. Frequency of various types of mutations in frequently mutated genes. Each type of mutation is indicated using a specific color.

Table 2. Gene ontology (GO) analysis on frequently mutated genes using DAVID database

Term	P value	Benjamini	FDR
hsa05213:Endometrial cancer	8.22E-06	7.44E-04	4.83E-04
hsa05230:Central carbon metabolism in cancer	1.89E-05	7.44E-04	4.83E-04
hsa05214:Glioma	2.01E-05	7.44E-04	4.83E-04
hsa05218:Melanoma	2.86E-05	7.93E-04	5.15E-04
hsa05215:Prostate cancer	6.67E-05	0.001481	9.61E-04
h_ctcfPathway:CTCF: First Multivalent Nuclear Factor	1.71E-04	0.012001	0.011315
hsa04919:Thyroid hormone signaling pathway	1.89E-04	0.003501	0.002271
hsa04071:Sphingolipid signaling pathway	2.23E-04	0.003537	0.002294
hsa05223:Non-small cell lung cancer	3.82E-04	0.005306	0.003442
hsa05161:Hepatitis B	4.60E-04	0.005679	0.003683
hsa05210:Colorectal cancer	5.17E-04	0.005735	0.00372
hsa05212:Pancreatic cancer	5.94E-04	0.005992	0.003887
hsa05220:Chronic myeloid leukemia	8.02E-04	0.007414	0.004809
hsa05222:Small cell lung cancer	0.001299	0.011095	0.007197
hsa05205:Proteoglycans in cancer	0.00154	0.012211	0.00792
h_arfPathway:Tumor Suppressor Arf Inhibits Ribosomal Biogenesis	0.003121	0.067488	0.063631
h_ptenPathway:PTEN dependent cell cycle arrest and apoptosis	0.003121	0.067488	0.063631
hsa04722:Neurotrophin signaling pathway	0.003489	0.025818	0.016747
GO:0050900~leukocyte migration	0.003736	0.453024	0.453024
h_igf1mtorPathway:Skeletal muscle hypertrophy is regulated via AKT/mTOR pathway	0.003856	0.067488	0.063631
GO:0038128~ERBB2 signaling pathway	0.004247	0.453024	0.453024
hsa04960:Aldosterone-regulated sodium reabsorption	0.004525	0.029356	0.019042
hsa05160:Hepatitis C	0.004662	0.029356	0.019042
hsa04068:FoxO signaling pathway	0.00476	0.029356	0.019042
hsa04550:Signaling pathways regulating pluripotency of stem cells	0.005381	0.031437	0.020392
h_eif4Pathway:Regulation of eIF4e and p70 S6 Kinase	0.005547	0.075825	0.071492
h_mtorPathway:mTOR Signaling Pathway	0.006499	0.075825	0.071492
GO:0007173~epidermal growth factor receptor signaling pathway	0.009037	0.773486	0.773486
hsa05221:Acute myeloid leukemia	0.009161	0.050843	0.032979
GO:0006661~phosphatidylinositol biosynthetic process	0.009669	0.773486	0.773486
hsa04150:mTOR signaling pathway	0.009803	0.051546	0.033435
hsa04370:VEGF signaling pathway	0.010802	0.051546	0.033435
hsa04151:PI3K-Akt signaling pathway	0.01087	0.051546	0.033435
hsa04210:Apoptosis	0.011145	0.051546	0.033435
h_metPathway:Signaling of Hepatocyte Growth Factor Receptor	0.01229	0.122903	0.11588
hsa05211:Renal cell carcinoma	0.012564	0.055785	0.036185
hsa04664:Fc epsilon RI signaling pathway	0.013302	0.056232	0.036475
hsa04662:B cell receptor signaling pathway	0.013678	0.056232	0.036475
hsa04917:Prolactin signaling pathway	0.014444	0.05726	0.037141
hsa05203:Viral carcinogenesis	0.015293	0.058537	0.03797
hsa05200:Pathways in cancer	0.01694	0.062678	0.040656
hsa04012:ErbB signaling pathway	0.021215	0.073588	0.047733

The Association between the Frequently Mutated Genes and Clinicopathologic Characteristics

We evaluated the association of three significant clinical traits, including lymph node metastasis, ER status, and grade of malignancy, with genes that are frequently mutated. The findings showed that among these genes, the TP53 gene has the most clinical importance, because this gene had a significant relationship with all characteristics ($p<0.05$), so that its frequency was significantly higher in malignant tumors compared to benign tumors, ER- tumors compared to ER+ tumors, and LN+ cases compared to LN-

cases, and this means that with the mutation of this gene, the severity of malignancy and the acuteness of dog mammary tumor increases ($p<0.05$) (Figures 2-4). In benign cases, there was a significant increase in the frequency of PIK3CA and SLC6A8 mutations compared to malignant cases ($p<0.05$).

Figure 5 shows the frequency of TP53 mutation in different histopathological subtypes of canine mammary tumors. It should be noted that 68.5% of mutations in this gene occurred in two malignant subtypes of simple carcinoma (56%) and complex carcinoma (12.5%), and 31% of mutations in this gene occurred in other rare malignant subtypes of canine mammary tumors (osteosarcoma/fibrosarcoma). This mutation did not occur in benign subtypes, including simple adenoma, complex adenoma, and benign mixed tumor (Figure 5).

Furthermore, we investigated the distribution of different types of TP53 mutations in histopathological tumor subtypes. In summary, missense mutations were the predominant type and occurred in all histopathological subtypes of canine mammary tumors. Furthermore, nonsense mutations and splicing mutations occurred merely in simple carcinoma and osteosarcoma/fibrosarcoma, respectively (Figure 6).

The Association between the Frequently Mutated Genes and Survival

The study showed that among the 18 studied genes, two genes, TP53 and FSIP2, had a significant relationship with the patient's survival, so that dogs with these gene mutations have a 2.8 (95% CI = 1.30-6.0, P = 0.009) and 2.5 (95% CI = 1.03-5.9, P = 0.041) times higher risk of death than dogs without these mutations, respectively. Also, the survival time in high-risk patients was significantly shorter than in low-risk patients ($P < 0.05$) (Figure 7A and Figure 8). Because TP53 mutations occurred only in malignant tumors, we also performed survival analysis in malignant cases separately. Again, we obtained similar findings, so that the survival time in the high-risk group (median survival time = 127 days) was considerably shorter than in the low-risk group (median survival time = 260 days) (P = 0.013) (Figure 7B).

Validation of the Frequently Mutated Genes Using CNV Data

Our analysis of array-CGH data showed that CNVs were present in the canine TP53 genomic region (chr5: 32665395-32678721) in 4 out of 12 tumor samples (33%). CNVs were characterized by chromosomal loss abnormalities. In addition, there was a significant association between CNV of frequently mutated genes and ER status, so that all cases containing CNV (100%) and 3 out of 8 cases without CNV (37%) were ER-negative (P = 0.038).

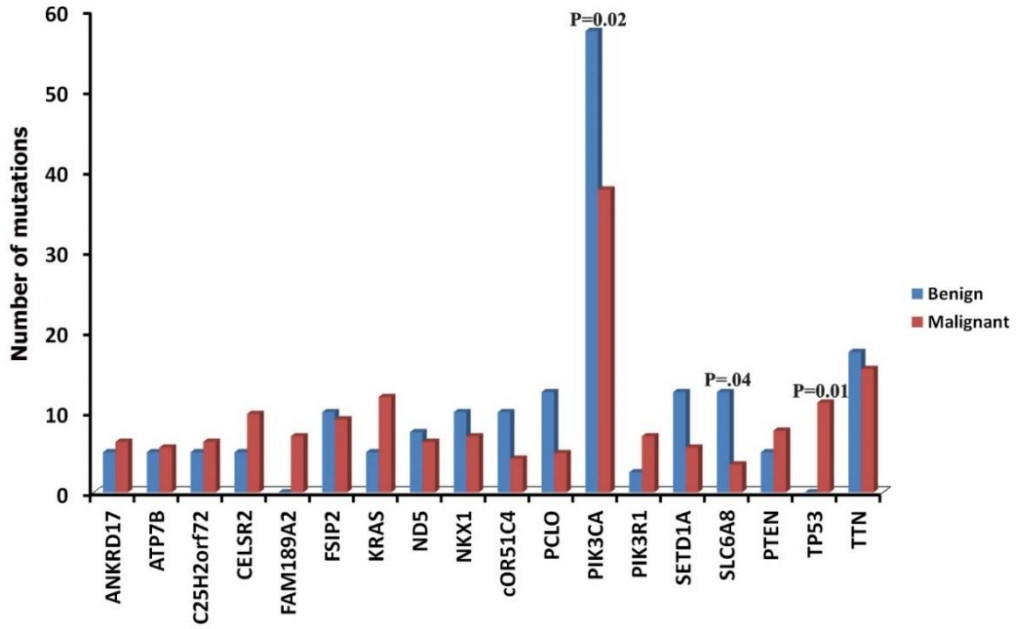

Figure 2. Association between frequency of frequently mutated genes and malignancy. Clearly, malignant cases with TP53 mutation were significantly more than benign cases (p = 0.01).

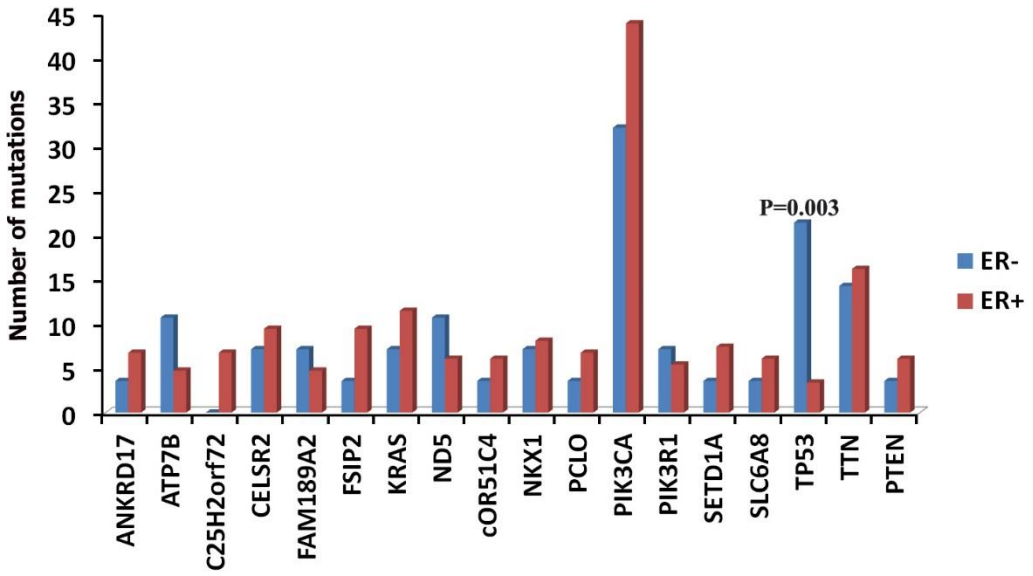

Figure 3. Association between frequency of frequently mutated genes and estrogen receptor (ER) status. The frequency of TP53 mutation was significantly higher in ER- patients than in ER+ patients (p = 0.003).

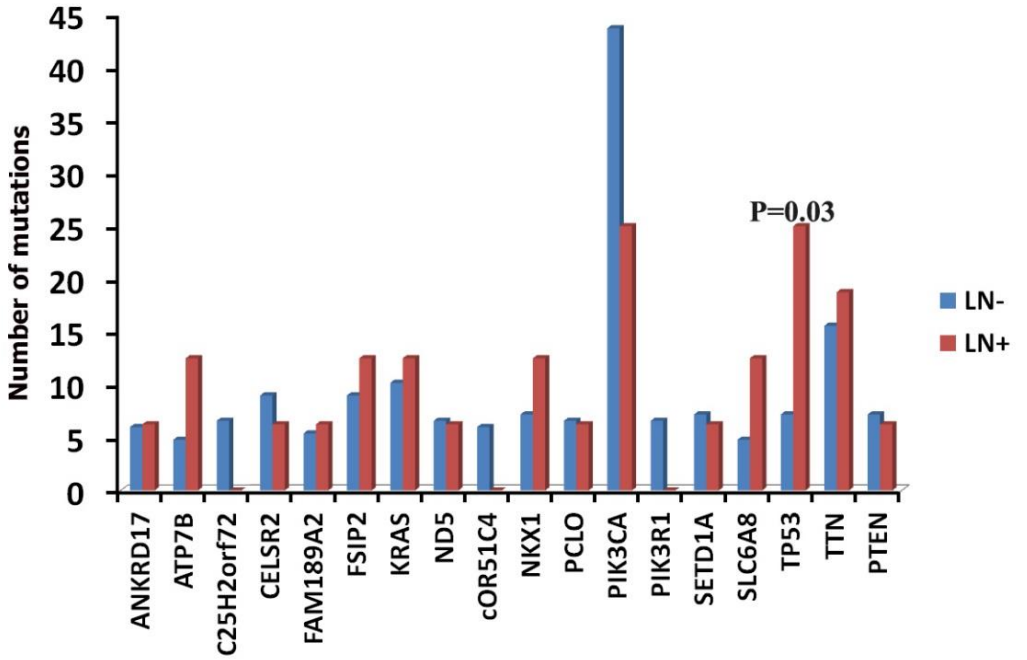

Figure 4. Association between frequency of frequently mutated genes and lymph node metastasis. The frequency of TP53 mutation were significantly higher in LN+ patients than in LN- patients (p = 0.03).

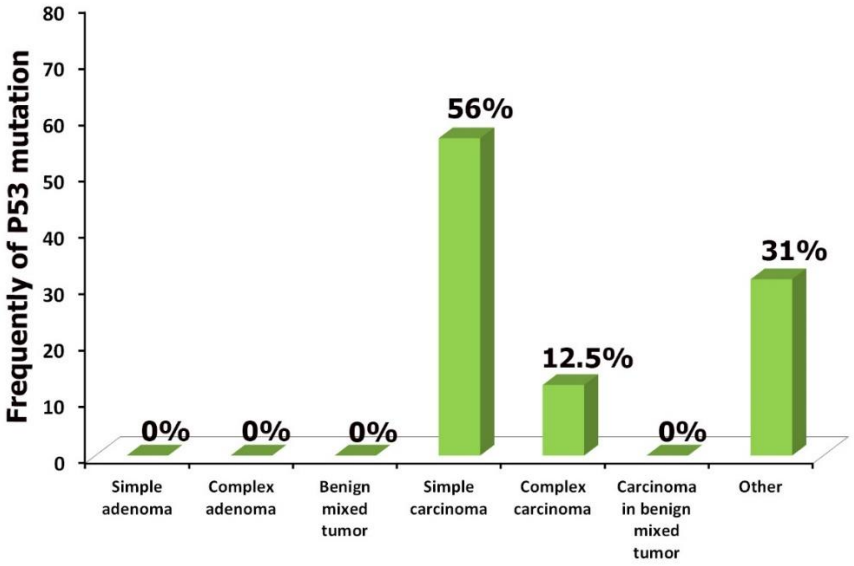

Figure 5. Frequency of TP53 mutation across different histopathological subtypes of canine breast cancer. TP53 frequently mutated in malignant subtypes (simple carcinoma and complex carcinoma) and other rare malignant subtypes of canine mammary tumors (osteosarcoma/fibrosarcoma).

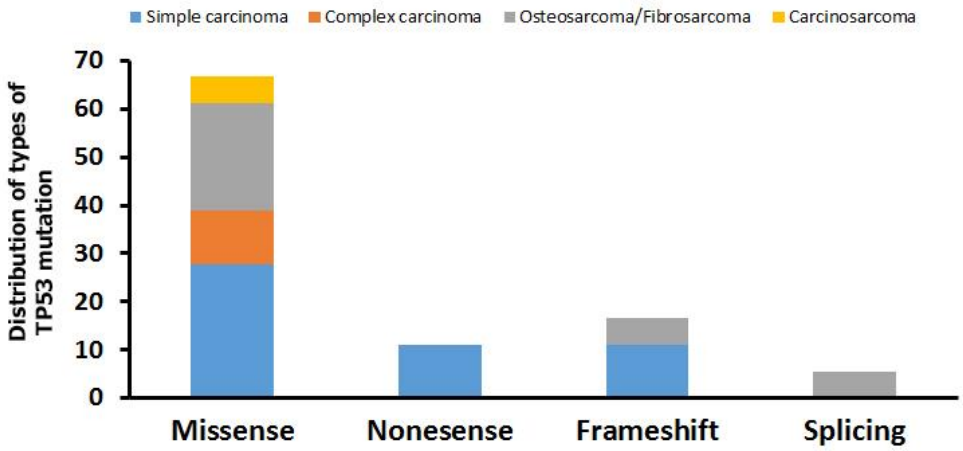

Figure 6. Distribution of various types of TP53 mutation between different histopathological subtypes of canine mammary tumor.

Discussion

In the present study, we investigated clinically important mutated genes in canine mammary tumors using DNA sequencing data. The most important result in our study was the crucial role of the TP53 gene in dog mammary tumors, as this gene was one of the most commonly mutated genes that had significant correlations with various clinical parameters. TP53 is a well-known gene in cancer biology with multiple functions as one of the most important tumor suppressors. This gene encodes the p53 protein in response to various cellular stresses. As a transcription factor, the p53 protein then regulates various basic cellular processes such as maintenance of genomic stability (DNA damage repair), cell division, cellular senescence, immune response, apoptosis, and autophagy (31,32). Like many other cancers in humans, TP53 mutations have been reported in breast cancer. TP53 is the most commonly mutated gene in breast cancer and is mutated in approximately 30% of cases (33). In general, using traditional methods such as PCR or more advanced sequencing techniques such as DNA sequencing, several studies have reported that patients with TP53 mutations have worse survival than healthy controls (14,34,35). In addition, in a study by Shiao et al., they showed that there was a racial difference between patients with TP53 mutation, so that TP53 mutation, which was associated with worse survival, was significantly more common in Black patients than in White patients (36). In addition, TP53 mutation can be used to predict therapeutic efficacy (37,38). For example, all patients who experienced

a pathologic complete response (PCR) in response to high-dose epirubicin and cyclophosphamide were shown to have a TP53 mutation (39). In another similar study, 36.5% of patients with TP53 mutant tumors showed a positive pCR compared to 0% of patients with wild-type tumors (40). There is a significant correlation between lymph node invasion or metastasis and TP53 mutation in breast cancer cases. This association increases the invasiveness and metastatic potential of breast cancer cells through different molecular pathways, such as the secretion of Rab-coupling protein, Hsp90, G-protein-coupled receptor, and adenosine receptor A2B (ADORA2B) (41,42). The frequency of TP53 mutation is significantly higher in high-grade malignant breast cancers than in low-grade ones (43). Also, breast cancer subtypes that are more aggressive have been reported to have a high rate of TP53 mutations (44).

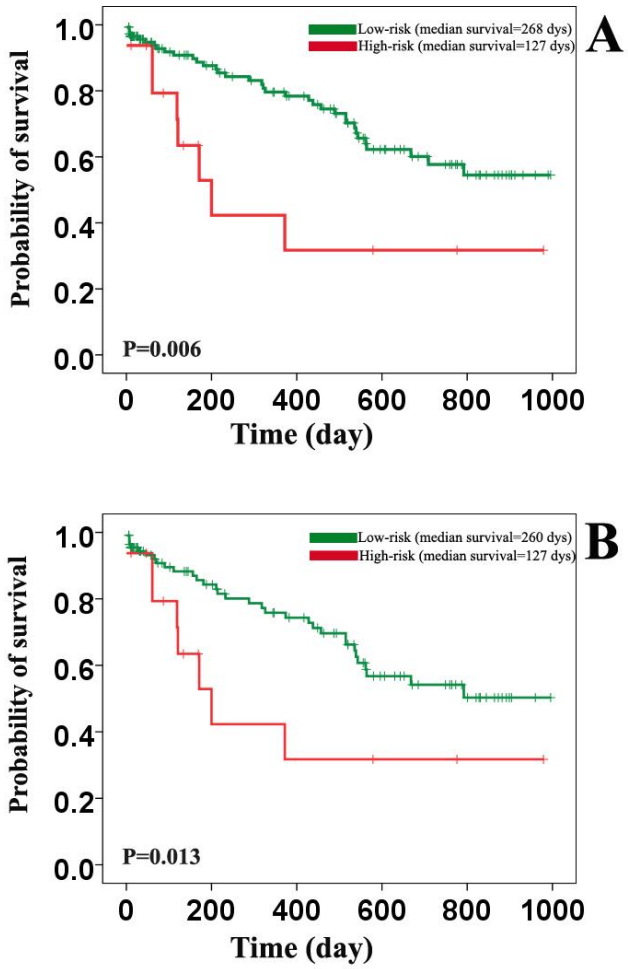

Figure 7. Survival analysis compared survival time between dogs with TP53 mutation (high-risk group) and dogs without TP53 mutation (low-risk group) in all cases (A) and in malignant cases (B). Cases with TP53 mutation significantly had lower survival time ($p<0.05$).

The clinical significance of TP53 mutations in canine mammary tumors has been evaluated in limited studies. However, in these studies, the TP53 mutation has been evaluated individually using traditional methods such as PCR and similar techniques. In agreement with our findings, most of these studies confirmed that TP53 mutation is an important prognostic factor indicating a higher risk of death in dogs with mammary tumors (16,45,46). These studies only investigated the association between TP53 mutation and survival time, and there is no information on the association between TP53 and other clinical features, such as lymphatic invasion/metastasis, ER status, and histological subtypes. Despite the fact that some researchers have reported TP53 mutations in benign tumors (16,46), we found that all TP53 mutations occur only in malignant samples.

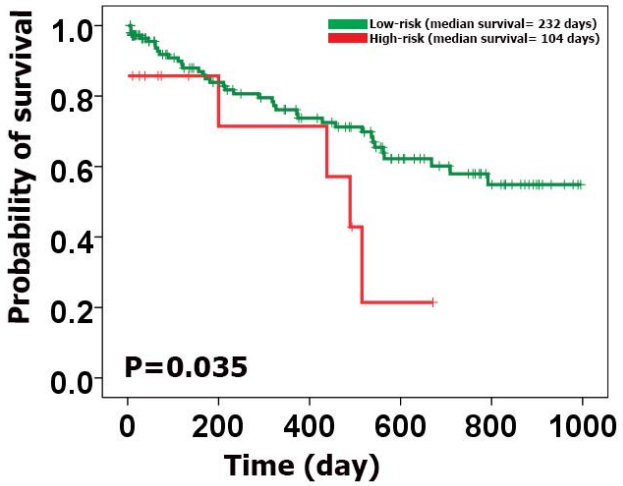

Figure 8. Survival analysis compared survival time between dogs with FSIP2 mutation (high-risk group) and dogs without FSIP2 mutation (low-risk group). Cases with FSIP2 mutation significantly had lower survival time ($p = 0.035$).

Another important gene that contained a large number of mutations in our study was PIK3CA. We found that the mutation rate of this gene in benign samples is considerably higher than in malignant tumors. Despite the high mutation rate, there was no significant relationship between the mutation of this gene and clinical characteristics such as survival and clinical outcome of patients. Similarly, PIK3CA was also considered a critical gene in human breast cancer. However, there are conflicting opinions regarding the PIK3CA mutation rate and survival of human breast cancer patients. While several studies have shown a positive correlation between high PIK3CA mutation rate and longer survival (47-50), other studies have revealed that a high rate of PIK3CA mutation is associated with worse outcome (51-54). In addition, some studies reported that this gene had no association with survival (55-57). These contradictory findings can be described by the heterogeneity parameters

of the studied populations, such as sample size, subgroups, and type of treatment (58).

Another gene that was significantly associated with survival in our study was FSIP2. Although the association of this gene with survival or outcome in human breast cancer has not been evaluated, mutations in FSIP2 have been found to occur significantly more often in metastatic breast cancers than in breast cancers in early stages (59).

Conclusion

Here, we conducted a study to find clinically relevant mutated genes in canine mammary tumors. With the development and expansion of the omics datasets on this canine tumor in the future, a clearer and more comprehensive view of the mutated genes will be depicted. In addition, using larger datasets and larger groups of dogs with mammary cancer, genes such as TP53 and other important genes identified in future studies can be investigated in more detail for different characteristics and especially for their clinical significance.

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Authors' Contributions

Mohammad Zamani Ahmadmahmudi: Conceptualization, Formal data analysis, Methodology, Investigation, Writing – original draft, Writing – review & editing, **M. Rezaei:** Investigation, review & editing

Data Availability

The data that support the findings of this study are available in SRA at https://www.ncbi.nlm.nih.gov/sra/?term=SRP159481, reference number SRP159481.

Ethical Approval

Not applicable

Conflict of Interest

None of the authors have any financial or personal relationships that could inappropriately influence or bias the content of the paper.

Consent for Publication

Not applicable.

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