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Mutations and polymorphisms in the mitochondrial genome of dogs with solid mammary carcinoma: A preliminary study*

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Summary

In recent years, the association between mitochondrial DNA (mtDNA) changes and tumorigenesis has been discussed. In contrast to human medicine, there are still few studies on the molecular basis of canine tumors. One of the most commonly diagnosed, solid mammary carcinoma, is characterized by its aggressiveness, difficult treatment, and generally poor prognosis. The aim of the study was to reveal mutations and polymorphisms in mtDNA in dogs with solid mammary carcinoma and to determine the relationship of these changes with the process of neoplastic transformation. Blood, healthy tissue, and neoplastic tissue samples were collected from two crossbreed dogs diagnosed with G3 and G2 solid mammary carcinoma. Subsequently, for the first time, Next Generation Sequencing (NGS) was used to analyze the whole genome mtDNA of dogs with solid mammary carcinoma. Thus, bioinformatic analyses included all 37 mitochondrial genes. As a result, 10 polymorphisms and 20 mutations were identified. Most polymorphisms/mutations were found in the dog with the more advanced stage of the disease (G3). Twelve of the thirty changes identified have not been described in the literature so far. These include eleven mutations in *COX2* (m.7308A>G), *ATP6* (m.8536C>T), *ND4L* (ins.9913_9914AG, ins.9913_9914TG, m.10165C>T), *ND4* (m.10204C>T), *CYTB* (m.16248A>G, m.16268A>G), D-loop (m.16378G>A, m.16408G>A, m.16507T>A) which may be related to canine tumorigenesis.

Keywords: mtDNA, dog disease, tumor, mutations, MGT

Canine cancer research has been increasingly popular in recent years. This is related not only to the increasing number of diagnoses, but also to the discovery of similarities in the aetiology of the disease at the molecular level and in response to cytotoxic agents in humans and dogs. In fact, some nuclear-derived biomarkers, such as *BRCA1* and *BRCA2* gene mutations, are clinically significant in human breast cancers (HBC) and are playing an important role in canine mammary tumors (CMT) (15, 18). Moreover, social development has led to the humanization of dogs, which are now regarded as family members (8).

One of the most common cancers in bitches is mammary carcinoma. There are many types of this disease. One example is solid mammary carcinoma, which is among the most common (11% of all diagnosed) and most difficult to treat, especially because it is not clearly separated from healthy tissue (4, 32). In order to predict the progression of the disease, mammary tumors have been characterized on the basis of, among others, the level of tubule formation, the number of tissue cells in mitosis, and differences in the structure of the nucleus. These characteristics make it possible to distinguish between three stages of malignancy: G1, G2, and G3. The lowest grade of malignancy, G1, is characterized by high tubule formation, low pleomorphism, and few mitoses, which is associated with the lowest propensity for recurrence and metastasis to distant organs. In contrast, the highest grade of malignancy, G3, is associated with the shortest patient life

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expectancy due to high nuclear pleomorphism, minimal tubular formation, and numerous mitoses (25). The origin of the disease is mostly unclear. However, one of the main risk factors for mammary gland tumors is the patient's sex, followed by age, breed, sterilization status and age at sterilization, as well as obesity and diet (11, 24, 33).

Mitochondria are responsible for numerous vital cellular functions, so even a small change in thousands of copies of mtDNA can result in multiple bodily dysfunctions, often characterized by a high mortality rate. There have been many scientific reports describing cancer-associated alterations in mitochondrial DNA (mtDNA) in humans. However, studies describing this association with cancer in dogs are still limited. Research on dogs began after 1998, when the mitochondrial genome of dogs was sequenced for the first time (16). According to data from The National Center for Biotechnology Information, the size of the mtDNA of *Canis lupus familiaris* is 16,727 bp. It encodes a total of thirty-seven genes, including 22 genes encoding tRNAs, 2 genes encoding rRNAs (12S, 16S), and 13 protein genes involved in oxidative phosphorylation: *ND1* (NADH dehydrogenase subunit 1), *ND2* (NADH dehydrogenase subunit 2), *COX1* (cytochrome c oxidase subunit I), *COX2* (cytochrome c oxidase subunit II), *ATP8* (ATP synthase F0 subunit 8), *ATP6* (ATP synthase F0 subunit 6), *COX3* (cytochrome c oxidase subunit III), *ND3* (NADH dehydrogenase subunit 3), *ND4L* (NADH dehydrogenase subunit 4L), *ND4* (NADH dehydrogenase subunit 4), *ND5* (NADH dehydrogenase subunit 5), *ND6* (NADH dehydrogenase subunit 6), and *CYTB* (cytochrome b). The only non-coding and triple-stranded region of mitochondrial DNA is the D-loop (Displacement loop). Compared to nuclear DNA, mitochondrial DNA is 10-20 times more susceptible to mutations, which is due to (a) errors accumulating in the replication process throughout the life of the organism, (b) the lack of protective activity of histones, (c) the absence of introns in the structure of mitochondrial genes, and (d) its proximity to the main source of reactive oxygen species of the cell (1, 12, 21). Despite the relatively short time that has elapsed since the canine genome was sequenced, researchers have managed to identify some links between mutations and/or polymorphisms in the mitochondrial genome and the occurrence of mammary gland tumors in dogs (19, 27-30). It is worth noting, however, that most studies do not include genome-wide analyses, but rather focus on detecting changes in a specific gene.

This is the first time that the entire mitochondrial genomes of two dogs with mammary solid carcinoma were analyzed. Furthermore, differences at the molecular level between malignancy degrees G2 and G3 were demonstrated. The need for this study is accentuated by the scarcity of scientific reports describing this type of cancer in dogs. Previous studies have analyzed differences at the immunohistopathological level, but there is still a lack of reports on molecular changes in mammary solid carcinoma. Therefore, the aim of the study was to reveal mutations and polymorphisms in mtDNA in dogs with solid mammary carcinoma and to determine the relationship of these changes with the process of neoplastic transformation.

Material and methods

Six samples were obtained from tumor tissues (n = 2), blood (n = 2), and healthy tissue (n = 2) of two twelve-year-old crossbreed female dogs with diagnosed solid mammary carcinoma. One with the malignancy grade G2 (lab. number: S08) and one with the malignancy grade G3 (lab. number: B114) were analyzed. Both dogs received neither hormone therapy nor chemotherapy. The dogs had not been sterilized either. DNA was extracted from post-operative tissues. The biological materials were collected according to Resolution number 79/2014 of the Second Local Ethical Committee for Animal Experiments in Lublin, Poland.

DNA extraction from all tissues was performed with a DNeasy Blood & Tissue Kit (Qiagen). DNA samples were assessed qualitatively by electrophoretic separation in 1.5% agarose gel and quantitatively (Nanodrop DeNovix DS-11, Thermo Fisher, Waltham, USA). Tumor tissue samples were placed in a sterile container. Blood was sampled into sterile test glasses with K3EDTA anticoagulant (Medlab, Raszyn, Poland).

In order to identify the types of the mammary tumor tissues collected, histopathological evaluation was performed by the Department of Pathomorphology and Forensic Medicine, Faculty of Veterinary Medicine, University of Life Sciences in Lublin, Poland. The mammary tumor tissues were fixed with 10% buffered formalin (pH 7.2) for 24 h and then passed through increasing concentrations of alcoholic solutions to acetone and xylene. After that, the tissues were embedded in paraffin blocks in a tissue processor (Leica TP-1020, Leica Biosystems, Nussloch, Germany). Next, 4 µm thick tissue sections were prepared with a sled microtome (Leica SR-200, Leica Biosystems, Nussloch, Germany) and placed on microscope slides. For histopathological evaluation, the preparations were stained with hematoxylin and eosin (HE) and evaluated under a light microscope (Olympus BX43, Tokyo, Japan) coupled with a digital camera (Olympus SC100, Tokyo, Japan). The malignancy degree of the solid mammary tumors was assessed using a 3-grade scale of malignancy, i.e., the sum of point values assigned to histomorphological traits according to Goldschmidt et al. (2011) (10).

Mitochondrial DNA was amplified on the total genomic DNA using two pairs of primers: F1418 and R11041, ~9.5kb PCR product, and 9190F and R2382, ~9.8kb PCR product (14). These two long-range PCR amplicons fully covered the mitochondrial genome sequence at least 900× (Tab. 1).

PCR reactions were carried out using KAPA HiFi PCR Kit reagents (KAPA Biosystems, Wilmington, USA). After amplification, the PCR products were analyzed in agarose gel and purified using Ampure XP magnetic beads (Beckman Coulter, Brea, USA). The quantity of DNA was measured with a Qubit 3.0 fluorimeter (Thermo Fisher Scientific, Waltham, USA). PCR products were diluted to the desired

Tab. 1. Detailed information on the length of PCR reads generated in the Illumina MiSeq

	Sample	Number of sequences	Sum of length	Average fragment length	Sequence cover
Read 1	B114T	89,930	19,211,555	213.6	2,022.27
	B114B	71,113	16,477,028	231.7	1,734.42
	B114H	47,153	10,335,744	219.2	1,087.97
	S08T	83,158	18,272,682	219.7	1,923.44
	S08B	42,887	8,873,173	206.9	934.02
	S08H	47,210	10,576,645	224.0	1,113.33
Read 2	B114T	89,930	19,442,845	216.2	1,983.96
	B114B	71,113	16,643,996	234.0	1,698.37
	B114H	47,153	10,421,079	221.0	1,063.38
	S08T	83,158	18,434,846	221.7	1,881.11
	S08B	42,887	8,961,091	208.9	914.40
	S08H	47,210	10,658,914	225.8	1,087.64

Explanations: T – tumor sample; B – blood sample; H – healthy tissue sample; B114, S08 – laboratory dogs' numbers

concentration and pooled in an equimolar ratio. Such a DNA mixture was then used in library preparation.

Mitochondrial DNA was sequenced by an external service. Approximately 1 ng of the PCR DNA template mix was mechanically sheared by nebulisation and an Illumina shotgun library was constructed using a TruSeq PCR-Free Kit (Illumina, San Diego, CA) according to the manufacturer's instructions. The library sample was sequenced on an Illumina MiSeq sequencer (Illumina, San Diego, CA) using a 600-cycle kit (v3) targeting a minimum 100× coverage. The sequence reads were filtered by quality with the FastX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and assembled into contigs with the Newbler v.3.0 GS De Novo Assembler (Roche, USA). Contig alignments were investigated using the Seqman software from a Lasergene package (DNAStar, USA). The remaining gaps were complemented by PCR and confirmed by Sanger capillary sequencing.

The sequences obtained were subjected to bioinformatic analyses. In order to identify mutations and polymorphic sites within the mtDNA genome, the Unipro UGENE v. 38.0 (23) and Canis mitoSNP (18) tools were used. Sequences were compared with the reference sequence deposited in GenBank (16). Polymorphisms included changes in both tumor and normal tissues, as well as in blood cells from the same test dog, compared to the reference sequence. A mutation, on the other hand, is a change that is specific to cancer cells and/or blood cells, but does not occur in all tissues tested in the same dog. ExPASy Server (9), SOPMA, and deepTMHMM (5) were used to perform protein analyses, such as predicting 2-row structures, as well as to characterize physico-chemical parameters, predict transmembrane helixes, etc. In describing variants of sequences found in the DNA and proteins, the HGVS (2016) nomenclature was used (7).

Results and discussion

In this study, a total of 14 polymorphisms and 34 mutations were identified in crossbreed dogs with solid mammary carcinoma of varying degrees of malignancy (Tab. 2 and 3). Alterations were detected in 8 of the 13 protein-coding mtDNA, and in the gene coding *tRNA-Leu (UUR)* (*MT-TL1*). However, changes were most frequent in the non-coding region of the D-loop. No mutations or polymorphisms were detected in five protein-coding genes (*ND1*, *ND2*, *ATP8*, *ND3*, *ND6*), 21 tRNA-encoding genes, and the 12S rRNA-encoding gene. Most of the changes were substitutions.

In relation to the reference sequence, polymorphisms in G3 cancer were detected in 8 positions of the mitochondrial DNA (Tab. 2). The highest number was identified in the *COX1* (m.5367C>T, m.5444T>C, m.6065A>G). Two out of the seven polymorphisms identified in CDS (coding DNA sequence of a protein coding gene) were non-synonymous, resulting in changes in the amino acid sequence. The polymorphism m.8807G>A in the *COX3* gene resulted in the substitution of cysteine for tyrosine (p.Cys55Tyr). In the *ND5* gene, the replacement of serine with threonine (p.Ser508Thr) resulted from the polymorphism m.13299T>A. Six polymorphisms were observed in the dog with G2 solid mammary carcinoma (Tab. 2). Four variants were the same as noted in the dog with the G3 stage of disease (ins.2678_2679G, m.5444T>C,

Tab. 2. Polymorphisms in mitochondrial genome sequences of the reference sequence, blood, healthy and tumor tissues from crossbreed dogs with solid mammary carcinoma of varying degrees of malignancy

Number of dog and grading	Gene	Reference sequence NC_002008.3	Sequences in blood, tumor cells and healthy tissue	Amino acid change	Position of the changed nucleotide in the codon
B114 G3	MT-TL1	m.2678_2679	m.2678_79insG*	–	–
	COX1	m.5367C	m.5367C>T*	p.Leu7=	CTG→TTG
		m.5444T	m.5444T>C*	p.Ala32=	GCT→GCC
		m.6065A	m.6065A>G*	p.Gly239=	GGA→GGG
	ATP6	m.8368C	m.8368C>T*	p.Leu135=	CTC→CTT
	COX3	m.8807G	m.8807G>A*	p.Cys55Tyr	TGC→TAC
	ND5	m.13299T	m.13299T>A*	p.Ser508Thr	TCA→ACA
	D-loop	m.15814C	m.15814C>T*	–	–
S08 G2	MT-TL1	m.2678_2679	m.2678_79insG*	–	–
	COX1	m.5444T	m.5444T>C*	p.Ala32=	GCT→GCC
		m.6065A	m.6065A>G*	p.Gly239=	GGA→GGG
	ATP6	m.8368C	m.8368C>T*	p.Leu135=	CTC→CTT
	ND4L	m.9913_9914	m.9913insGT	p.Met1Val	ATG→GTG
	D-loop	m.16148A	m.16148A>G*	–	–

Explanations: * – changes described in a review by Tkaczyk-Wlizło et al. (2022) (position 31 in the bibliography); "–" – not applicable

Tab. 3. Mutations in mitochondrial genome sequences of the reference sequence, blood, healthy and tumor tissues from crossbreed dogs with solid mammary carcinoma of varying degrees of malignancy

Number of dog and tumor grading	Gene	Reference sequence NC_002008.3	Sequences in blood	Sequences in tumor cells	Sequences in healthy tissue	Amino acid change	Position of the changed nucleotide in the codon
B114 G3	MT-TL1	m.2683G	m.2683G>A*	m.2683G>A*	m.2683G	–	–
	COX2	m.7308A	m.7308A	m.7308A	m.7308A>G	p.Asn92Ser	AAC→AGC
	ATP6	m.8536C	m.8536C>T	m.8536C>T	m.8536C	p.Ile191Ile	ATC→ATT
	ND4L	m.9913_9914	m.9913insGT	m.9913insGA	m.9913_9914	p.Met1Val p.Met1Glu	ATG→GTG ATG→GAG
		m.10165C	m.10165C>T	m.10165C>T	m.10165C	p.Ala67Val	GCT→GTT
	ND4	m.10204C	m.10204C	m.10204C	m.10204C>T	p.Cys98=	TGC→TGT
	CYTB	m.14474G	m.14474G>A*	m.14474G>A*	m.14474G	p.Val66Ile	GTC→ATC
	D-loop	m.15639T*	m.15639T>A*	m.15639T>A*	m.15639T	–	–
		m.16025T	m.16025T>C*	m.16025T>C*	m.16025T	–	–
		m.16148A	m.16148A	m.16148A>G*	m.16148A	–	–
		m.16168A	m.16168A	m.16168A>G*	m.16168A	–	–
		m.16248A	m.16248A	m.16248A	m.16248A>G	–	–
		m.16268A	m.16260_16299del(CGTACACGTAC)$_4$	m.16268A	m.16268A>G	–	–
		m.16368G	m.16368G	m.16368G>A*	m.16368G	–	–
		m.16378G	m.16378G	m.16378G>A	m.16378G	–	–
		m.16408G	m.16408G	m.16408G>A	m.16408G	–	–
		m.16507T	m.16507T	m.16507T	m.16507T>A	–	–
S08 G2	D-loop	m.16138A	m.16138A	m.16138A>G	m.16138A	–	–
		m.16398A	m.16398A>G*	m.16398A	m.16398A>G*	–	–
		m.16408G	m.16408G>A	m.16408G	m.16408G>A	–	–
		m.16418A	m.16418A>G*	m.16418A	m.16418A>G*	–	–

Explanations: * – changes described in a review by Tkaczyk-Wlizło et al. (2022) (position 31 in the bibliography); "–" – not applicable

m.6065A>G, m.8368C>T). All of them were synonymous.

Mutations were detected in seven regions of the mitochondrial genome (Tab. 3). More alterations were observed in tumor with the malignancy degree G3 than in G2. Most of them were transversions, two were transitions (m.15639T>A, m.16507T>A), and two were insertions (ins.9913_9914AG, ins.9913_9914TG). The highest number of mutations were noted in the mitochondrial non-coding region (D-loop). In the dog with G2 cancer, four mutations (m.16138A>G, m.16398A>G, m.16408G>A, m.16418A>G) were detected only in the D-loop region (Tab. 3).

In the crossbreed dog with G3 solid mammary carcinoma, two deletions of a VNTR motif were revealed. In this dog's blood sample, the deletion involved a total of 40 nucleotides, including three 10-nucleotide repeat motifs 5'-GTACACGTAC-'3 and one 5'-GTACACGTGC-'3. In addition, in healthy tissue, deletions of one 5'-GTACACGTAC-'3 VNTR motif were observed. Regarding the crossbreed dog with G2 solid mammary carcinoma, only one deletion of the VNTR motif 5'-GTACACGTAC-'3 was detected in the tumor cells (Tab. 7).

Analyses of protein properties in the non-synonymous protein-coding SNP of *COX2*, *COX3*, *ND5*, and

Tab. 4. Comparison of protein properties in non-synonymous protein-coding SNP calculated by the ExPASy Server

Protein	Amino acid change	Theoretical pI (Isoelectric point)	Aliphatic index	Instability index	Grand average of hydropathicity (GRAVY)
COX2	p.Asn92Ser	4.62	109.91	38.70 (stable)	0.308
	Normal	4.62	109.91	36.64 (stable)	0.296
COX3	p.Cys55Tyr	6.44	93.37	20.87 (stable)	0.444
	Normal	6.44	93.37	19.67 (stable)	0.459
ND4L	p.Met1Val	5.27	143.16	38.74 (stable)	1.326
	p.Met1Glu	4.80	140.20	40.71 (unstable)	1.247
	p.Ala67Val	5.27	142.14	43.22 (unstable)	1.327
	Normal	5.27	140.20	43.22 (unstable)	1.302
ND5	p.Ser508Thr	9.28	109.95	31.07 (stable)	0.591
	Normal	9.28	109.95	31.51 (stable)	0.591
CYB	p.Val66Ile	6.85	121.21	38.61 (stable)	0.716
	Normal	6.85	120.95	38.39 (stable)	0.715

Tab. 5. Secondary structure elements calculated by SOPMA

Protein	Secondary structure	Alpha helix (%)	310 helix (%)	Pi helix (%)	Beta bridge (%)	Extended strand (%)	Beta turn (%)	Bend region (%)	Random coil (%)	Ambiguous states (%)	Other states (%)
COX2	p.Asn92Ser	31.28	0	0	0	23.35	4.85	0	40.53	0	0
	Normal	31.28	0	0	0	25.11	5.29	0	38.33	0	0
COX3	p.Cys55Tyr	50.19	0	0	0	15.71	5.75	0	28.35	0	0
	Normal	48.28	0	0	0	17.62	6.90	0	27.20	0	0
ND4L	p.Met1Val	72.45	0	0	0	8.16	2.04	0	17.35	0	0
	p.Met1Glu	78.57	0	0	0	7.14	2.04	0	12.24	0	0
	p.Ala67Val	76.53	0	0	0	6.12	3.06	0	14.29	0	0
	Normal	84.69	0	0	0	6.12	3.06	0	6.12	0	0
ND5	p.Ser508Thr	53.80	0	0	0	14.03	3.80	0	28.38	0	0
	Normal	53.96	0	0	0	14.36	4.29	0	27.39	0	0
CYB	p.Val66Ile	50.40	0	0	0	10.55	4.22	0	34.83	0	0
	Normal	51.19	0	0	0	12.14	4.22	0	32.45	0	0

CYTB genes showed no significant discrepancies between the reference sequence and the altered sequences (Tab. 4). Secondary structure elements are presented in Tab. 5. However, the insertion of m.9913insGT in the *ND4L* protein may have a potentially detrimental effect on its function by changing the stability index from unstable (43.22) to stable (38.74). At the same time, no significant differences were observed for other changes in *ND4L*. According to the results, alpha helix dominated in the secondary structure of the *COX3* (p.Cys55Tyr), *ND4L* (p.Met1Val, p.Met1Glu, p.Ala67Val), ND5 (p.Ser508Thr), and *CYTB* (p.Val66Ile), followed by random coil, extended strand, and beta turns. Only in *COX2*, was the random coil the dominant structure. The largest decrease in the percentage of the compact alpha helix structure (72.45%) was found in the case of *ND4L* (p.Met1Val) relative to normal protein (84.69%) (Tab. 5). The results revealed that non-synonymous mutations resulting in changes in amino acids (p.Cys55Tyr, p.Met1Val, p.Met1Glu, p.Ala67Val, p.Ser508Thr, p.Val66Ile) in the solid mammary carcinoma did not affect the number of transmembrane sequences (Tab. 6). The predicted number of amino acids in transmembrane helixes in the first 60 amino acids of the protein (Exp number, first 60 AAs) was comparable to the reference protein and *COX2*, *COX3*, *ND4L*, and *ND5* proteins with amino acid changes (Tab. 6).

Solid mammary carcinomas in dogs are malignant and aggressive tumors. In 2021, Borghesi et al. presented a basic immunohistopathological characteriza-

Tab. 6. Transmembrane helices in genes prediced by the Hidden Markov Model (TMHMM)

Protein	Amino acid change	Length	Number of predicted transmembrane helices	TheExp number of AAs in TMHs	Exp number, first 60 AAs
COX2	p.Asn92Ser	227	2	43.19579	21.0122
	Normal	227	2	43.26451	21.03997
COX3	p.Cys55Tyr	261	5	135.52792	31.186
	Normal	261	5	131.96199	27.46577
ND4L	p.Met1Val	98	3	62.85562	41.96735
	p.Met1Glu	98	3	62.85458	41.96555
	p.Ala67Val	98	3	62.78571	41.82742
	Normal	98	3	62.77297	41.8202
ND5	p.Ser508Thr	606	14	312.23238	40.00414
	Normal	606	14	312.2435	40.00415
CYB	p.Val66Ile	379	9	193.75424	22.91164
	Normal	379	9	193.39725	22.88697

Tab. 7. Variation in the number of tandem repeat motifs between the reference sequence, tumor cells, blood, and healthy tissue from crossbreed dogs with solid mammary carcinoma of varying degrees of malignancy

		Number of 10-nucleotide motifs in the VNTR region			
		5'-GTACACGTAC-'3	5'-GTACACGTGC-'3	5'-GTACACGTA/GC-'3	Deletions
	Reference sequence	16/30	14/30	0/30	0
B114	Sequences in tumor cells	17/30	13/30	0/30	0
	Sequences in blood	13/30	13/30	0/30	del.[GTACACGTAC]4
	Sequences in healthy tissue	13/30	16/30	0/30	del.[GTACACGTAC]
S08	Sequences in tumor cells	13/30	16/30	0/30	del.[GTACACGTAC]
	Sequences in blood	14/30	16/30	0/30	0
	Sequences in healthy tissue	14/30	16/30	0/30	0

tion of canine solid mammary tumors. As a result, initial proteins involved in the aggressiveness of these tumors were identified (4). Another study performed by Veena et al. in 2014 included 24 samples of canine mammary gland tumors, including two solid mammary tumors. The analyses yielded inconclusive results; one out of two solid tumors were found immunoreactive for p53 protein (22, 34). There are only few reports about molecular changes on the protein level in the biology of solid tumors, and molecular factors related to their type of tumorigenesis are still unknown. This study is the first report on changes in mtDNA in canine solid carcinoma that make it possible to identify specific polymorphisms and mutations. It is also worth noting that this is the first application of NGS for the examination of entire mtDNA genomes of dogs diagnosed with mammary solid carcinoma.

Changes in mtDNA seem to be significant in the development of cancer. This is caused, among others, by changes in genes encoding the proteins of the respiratory chain complexes, which affect the formation of reactive oxygen species and block the apoptosis of altered cells (13, 30). The available publications are partly focused on the non-coding region of mtDNA, whose mutations and polymorphisms are believed to be related to damage in the DNA structure and progression of mammary cancer. This is explained by the fact that this non-coding region plays an important role in transcription and replication processes (3, 28, 29). Seven of the sixteen changes in the D-loop detected in this study have already been described in the literature. They were identified in mtDNA positions m.15639T>A (17, 19, 26, 28), m.16025T>C (19, 26, 28), m.16148A>G (17, 19), m.16168A>G (17, 19), m.16368G>A (17, 19), m.16398A>G (19), m.16418A>G (17, 19), and m.15814C>T (19, 28) (Tab. 2, Tab. 3) in dogs with mammary tumors, such as tubulopapillary, complexus, comedo, and solidum carcinomas (17, 19, 26, 28). This may confirm the hypothesis about the importance of changes in the D-loop in carcinogenesis.

Various versions of variable number tandem repeats (VNTR) 5'-GTACACGTA/GC-'3, are associated with multiple disorders, such as idiopathic megaesophagus in German shepherd dogs (2). This fragment is located in the 16130-16430 bp region of the canine D-loop. In this study, mitochondrial genomes of dogs with different degrees of malignancy were examined, in which a variable number of VNTR regions were noted. Five VNTR regions with an altered number of motifs were found in the dog with G3 solid mammary carcinoma (del.[GTACACGTAC]4 in blood, del. GTACACGTAC in healthy tissue) and one deletion in tumor cells was detected in the dog with G2 tumor (del. GTACACGTAC) (Tab. 7). Changes in this region were also described in dogs diagnosed with tubulopapillary carcinoma and other mammary tumors (17). Authors showed that among those 30 tandem repeats in blood, 12 retained the 5'-GTACACGTAC-'3 motif, whereas 11 were present in tumor tissue. Additionally, a reduced number of 5'-GTACACGTGC-'3 repeats were observed in both blood and tumor. Interestingly, in the tumor tissue, there was also an increased number of 5'-GTACACGTA/GC-'3 motifs, which indicated heteroplasmy (17). Recent studies have shown a possible link between a variable number of motifs in VNTR and an increased risk of breast cancer in women (6). It is worth noting that canine VNTR regions are different from those found in humans. However, they might also be associated with the development of mammary tumors and genomic instability in these animals. This confirms the importance of further research on this region because of its potential association with tumorigenesis.

Nine out of ten polymorphic *loci* have been described in the literature as a change associated with the canine neoplastic process: ins.2678_2679G, m.5367C>T, m.5444T>C, m.6065A>G, m.8368C>T, m.8807G>A, m.13299T>A, m.15814C>T, m.16148A>G (17, 30). The above-mentioned polymorphisms were found in different types of canine mammary gland tumors. Thus, these changes are not characteristic of specific tumor types. It is worth noting that the insertion ins.9913_9914AG in the *ND4L* gene, as well as its nonsynonymous effect (p.Met1Glu) in the *ND4L* protein were identified for the first time. However, the polymorphism m.9911A>G was present in a Labrador Retriever with mammary gland tumor described by Kowal et al. in 2019 (17). A bioinformatic analysis of basic protein parameters (Tab. 4-6) revealed a change in the stability index for the insertion m.9913insGT in the *ND4L* protein, which may have a potentially detrimental effect on its function. The analysis of the remaining changes did not show remarkable differences in the values of parameters examined.

Non-coding RNA molecules (miRNA, tRNA, piRNA, incRNA, circRNA) are responsible for regulating numerous biological pathways and processes of the cell (35). There are still not enough studies to confirm the role of mutations occurring in their region in the process of canine cancerogenesis. However, in the present study, changes in the *tRNA-Leu* (*UUR*) gene were found in both animals (polymorphism m.2678_79insG in both dogs, mutation m.2683G>A in dog S08, and polymorphism m.5367C>T in dog B114). It is worth noting that a study conducted by Kowal et al. in 2022 revealed the aforementioned polymorphism in all 27 samples examined (from dogs with mammary gland tumors) and substitution m.2683G>A in 18, that is, almost 67% of all samples (20). This suggests a possible link between these changes and the process of carcinogenesis in dogs.

Noteworthy, eleven of the twenty detected mutation *loci* associated with the neoplastic process in dogs have not been described in the literature so far – including those in protein coding regions *COX2* (m.7308A>G), *ATP6* (m.8536C>T), *ND4L*

(ins.9913_9914AG, ins.9913_9914TG, m.10165C>T), and *ND4* (m.10204C>T). Significantly more mutations occurred in the dog with G3 tumor (25) than in the animal with G2 tumor (7). Furthermore, six lesions were characterized as somatic variants (variants observed only in cancer tissue samples). Among these, five variants (m.16148A>G, m.16168A>G, m.16368G>A, m.16378G>A, m.16408G>A) occurred in tissues from the dog diagnosed with G3 solid carcinoma, and one (m.16138A>G) was found in the animal with G2 solid carcinoma. It may indicate that the increasing number of alterations might be linked with a higher malignancy grade of solid carcinoma in dogs.

In conclusion, twelve mutations associated with canine solid carcinoma were described for the first time: *COX2* (m.7308A>G), *ATP6* (m.8536C>T), *ND4L* (ins.9913_9914AG, ins.9913_9914TG, m.10165C>T), *ND4* (m.10204C>T), *CYTB* (m.16248A>G, m.16268A>G), D-loop (m.16378G>A, m.16408G>A, m.16507T>A). Moreover, there was a noticeable relationship between the number of alterations and the degree of malignancy. The results suggest that there is need to continue research on a larger cohort of affected and control dogs.

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