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Mitochondrial DNA alternations in formalin-fixed paraffin-embedded tissue collected from dogs with malignant mammary gland tumours

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Formalin-fixed paraffin embedded (FFPE) tissue is the most common histopathological technique for tumour assessment in the veterinary pathology. Due to easy availability of FFPE blocks, the aim of this retrospective study was to identify molecular defects caused by mutations in mitochondrial DNA (mtDNA) in cases of canine malignant mammary gland tumours (MGTs). DNA was isolated from 88 FFPE samples, then quantity and quality analyses were performed. Amplification was conducted on 10 selected fragments of 8 mitochondrial genes (*COX1, COX3, ND1, ND2, ND4, ND4L, ND5* and *ATP6*), and finally obtained PCR products were sequenced. After that, bioinformatic analyses of nucleotide and protein sequences were done. The total number of observed mtDNA changes was 512 based on 23 changed nucleotide positions including 20 substitutions, 1 indel mutation and 4 heteroplasmic sites. The most commonly occurred mtDNA variants were m.8807G>A (*COX3*) and

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m.9911_9912insGT (*ND4L*) followed by m.3196T>C (*ND1*), m.5444T>C (*COX1*) and m.3254G/A (*ND1*). The changes in the protein-coding genes were mostly synonymous, and nonsynonymous changes were mostly tolerant and did not lead to alterations in protein properties. The number of mtDNA changes per sample varies from 2 to 13, and not depended on histopathological tumour type, tumour grading, age or breed of dog.

KEY WORDS: dog / neoplasms / FFPE / mtDNA / MGT

Companion animals, especially dogs, are treated as true family members nowadays. The attention devoted to the animals and their veterinary care resulted in their extended longevity and more profound insight into veterinary medicine. Therefore, the prevention, diagnosis and treatment of different canine diseases, including neoplasms, was improved [Rueda *et al.* 2024].

Currently, various types of neoplasms are observed in dogs. Among them, the most frequently observed are mammary gland tumours (MGTs). MGTs commonly affect bitches representing up to 50% of all types of neoplasms [Collivignarelli *et al.* 2021]. It is worth emphasising that MGTs are also reported rarely in male dogs [Tkaczyk-Wlizlo *et al.* 2024]. Depending on a study, the malignancy rates vary from 40% to 60% [Zappulli *et al.* 2019]. The prognostic evaluation of canine tumourigenesis includes tumour size and radiographic evidence of metastases combined with lymphatic and vascular invasion [Goldschmidt *et al.* 2011, Kaszak *et al.* 2022].

Histology is a standard in the diagnosis of canine MGTs. Microscopic evaluation, following appropriate staining, allows assessment of tumour type, benign or malignant nature, and grading in malignant neoplasms. With this ability, histopathological evaluation is an important step in prognostic diagnosis [Zappulli *et al.* 2019]. The most common histological technique of tumour tissue assessment in veterinary pathology is preparation of formalin-fixed paraffin embedded (FFPE) blocks. FFPE is a key diagnostic approach, as numerous MGT tissue blocks can be preserved for further analyses, including retrospective research. [Di Giacomo *et al.* 2022].

Despite years of research on the aetiopathogenesis of MGTs in dogs, their molecular background remains incompletely understood. In addition to changes in nuclear DNA (nDNA) [Borge *et al*. 2011], mitochondrial DNA (mtDNA) instability has also been observed in dogs with malignant MGTs. [Tkaczyk-Wlizlo *et al.* 2022]. Therefore, the aim of the study was to determine the molecular defects caused by mutations in 10 selected fragments of mtDNA using sequencing of malignant samples of MGTs in form of FFPE.

Material and methods

Ethical issue

All tumour samples were surgically resected during the treatment of dogs with mammary gland neoplasms. The collected biological materials were submitted to histopathological evaluations. No other additional biological samples were obtained

from animals. Due to that, an approval from Local Ethical Committee for Animal Experiments was not needed.

Sample information

Samples of mammary gland tumours were collected from 2020 to 2023. The retrospective study included 56 female dogs of different breeds such as Cane Corso, Dachshund, French Bulldog, German Shepherd, Pekingese, Yorkshire Terrier, and 32 female crossbreeds diagnosed with malignant mammary gland tumours. The available individual characteristics, including the breed, age, tumour type, and tumour grading of the affected female dogs are presented in Table 1.

Histopathological evaluations

The obtained post-operative tumour tissue was evaluated histopathologically. Tissue was routinely fixed with 10% buffered formalin (pH 7.2), passed through increasing concentrations of alcoholic solutions to acetone and xylene, and embedded in paraffin blocks in a tissue processor (Leica TP-1020, Leica Biosystems, Nussloch, Germany). From each formalin-fixed paraffin-embedded tumour block, 4-µm-thick sections were performed with a sled microtome (Leica SR-200, Leica Biosystems, Nussloch, Germany) and stained with haematoxylin and eosin (H&E). Histopathological evaluations were performed under a light microscope coupled with a digital camera (Olympus BX43, Olympus SC100, Tokyo, Japan) in accordance with the WHO histological recommendations [Misdorp *et al.* 1999] complemented by the latest criteria published by Zappulli *et al.* [2019]. Histopathological classification of malignant neoplasms of the epithelial origin and their grading was based on the classification system developed by Goldschmidt *et al.* [2011]. Grading of histopathological malignancy includes: GI – low, GII – intermediate, and GIII – high grade of malignancy. To assess grades of malignancy, summing point values assigned to tumour criteria such as cellular and nuclear pleomorphisms, tubule formation, random necrotic foci and mitotic index were included [Goldschmidt *et al.* 2011].

Molecular analysis

To eliminate overload of paraffin during DNA isolation, the excess of wax was removed by with a surgical blade. Then DNA was extracted using a QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration of isolated DNA samples was measured using a NanoDrop™ One/OneC Microvolume UV-Vis spectrophotometer (Thermo Scientific, Waltham, MA, USA) and the quality was assessed by electrophoretic separation in 1,5% agarose gel (multiSUB™ Maxi, Cleaver Scientific Ltd, UK).

Based on available literature, the most frequently altered regions of mitochondrial genomes in MGTs [Kowal *et al.* 2022, Kowal *et al*. 2023, Tkaczyk-Wlizlo *et al.* 2022] were identified, leading to the selection of 10 fragments, including the COX1, COX3, ND1, ND2, ND4, ND4L, and ND5 mtDNA genes. Primers of the indicated regions

Table 1. List of analysed samples of canine MGTs embedded in paraffin blocks

No	Breed of the dog according to FCI	Age (years)	Tumour type	Grading	Tumour origin	No	Breed of the dog according to FCI	Age (years)	Tumour type	Grading	Tumour origin
1	American Eagle	12	tubulopapillary carcinoma	GI	Epithelial	45	English Cocker Spaniel	6	solid carcinoma	GII	Epithelial
2	Australian Shepherd	8		GIII		46	French Bulldog	4	solid carcinoma	GI	
3	Bavarian Mountain Hound	8,5		GI		47		6	tubulopapillary carcinoma	GI	
4	Beagle	13		GI		48	German Shepherd	6	inflammatory carcinoma	GI	
5	Cane Corso	9	complex carcinoma	GI		49		7	complex carcinoma	GI	
6		9	tubulopapillary carcinoma	GI		50		9		GI	
7	Chinese Crested Dog	14	tubulopapillary carcinoma	GII		51		9	solid carcinoma	GIII	
8	Chow-Chow	10	complex carcinoma	GII		52		10	inflammatory carcinoma	GIII	
9	Cocker Spaniel	12	solid carcinoma	GII		53		10	solid carcinoma	GII	
10	Crossbreed	4	tubulopapillary carcinoma	GIII		54		10		GIII	
11		6	solid carcinoma	GI		55		10.5		GII	
12		7	complex carcinoma	GII		56		11	complex carcinoma	GI	
13		7		GII		57		12	solid carcinoma	GII	
14		8	tubulopapillary carcinoma	GIII		58		13	complex carcinoma	GII	
15		8		GIII		59	Giant Schnauzer	12	complex carcinoma	GII	
16		9	complex carcinoma	GII		60	Golden Retriever	11	complex carcinoma	GI	
17		9	solid carcinoma	GII		61	Gordon Setter	6	complex carcinoma	GI	
18		9		GIII		62	Great Dane	3	solid carcinoma	GII	
19		10	complex carcinoma	GI		63	Labrador Retriever	8	complex carcinoma	GI	
20		10		GII		64		13		GII	
21		10		GII		65	Maltese	10	complex carcinoma	GI	
22		10	tubulopapillary carcinoma	GIII		66		10	tubulopapillary carcinoma	GI	
23		10		GI		67		14	solid carcinoma	GII	
24		11	complex carcinoma	GI		68	Miniature Pinscher	11	tubulopapillary carcinoma	GIII	
25		11	solid carcinoma	GII		69	Miniature Schnauzer	9	complex carcinoma	GI	
26		11		GII		70		10	tubulopapillary carcinoma	GIII	
27		11		GII		71		12		GII	
28		11		GIII		72	Norwegian Elkhound	14	complex carcinoma	GII	
29		11	tubulopapillary carcinoma	GI		73	Pekingese	9	complex carcinoma	GII	
30		11		GII		74		10	solid carcinoma	GII	
31		11		GII		75	Polish Hunting Dog	10	complex carcinoma	GI	
32		11		GIII		76	Prague Ratter	12	tubulopapillary carcinoma	GII	
33		12	comedocarcinoma	GIII		77	Rottweiler	10	comedocarcinoma	GIII	
34		12	complex carcinoma	GI		78	Shih Tzu	7	complex carcinoma	GI	
35		12		GI		79	Siberian husky	10	tubulopapillary carcinoma	GI	
36		12		GII		80		10,5	tubulopapillary carcinoma	GI	
37		12	solid carcinoma	GIII		81	Terrier *	9	complex carcinoma	GI	
38		12	tubulopapillary carcinoma	GII		82	Weimaraner	12	tubulopapillary carcinoma	GII	
39		13	complex carcinoma	GI		83	West Highland White Terrier	10	complex carcinoma	GI	
40		14		GII		84	Yorkshire Terrier	10	complex carcinoma	GI	
41		16	solid carcinoma	GII		85		10		GI	
42	Dachshund	5	carcinoma solidum	GII		86		11		GI	
43		12	solid carcinoma	GIII		87		12	solid carcinoma	GII	
44		13		GIII		88		12,5	complex carcinoma	GI	

*Breed of terrier was not indicated.

Table 2. List of designed primers using Primer3 applied in study

	Gene name	Primers sequences	Range	Length (bp)
1	*COX1*	CCACGGAGCTTGGCAAAA TGGAGGAAGGAGTCAGAAGC	5271-5671	400
2		CTGATTCTTCGGACATCCTGA GCGATAATTATAGTGGCGGACG	6064-6287	223
3		AGACACACGAGCGTACTTTAC CTGTTAACCCGCCTACTGTAAA	6246-6412	167
4	*COX3*	GCCCTTTCTGCCCTCCTTAT GCTCAGAAGAAGCCTGCAAA	8705-8943	239
5	*ND1*	AGCCTCAAACTCCAAATACGC GGGGCTCGATTAGTTTCTGC	3110-3338	228
6	*ND2*	TCCGTTCTAGTAGGAGGCTG GGGATAAAGATAGGGTCGTGGT	4395-4608	214
7	*ND4*	CACAACATGACTTCTGCCCC AGAGTCCTGCGTTTAGTCGT	10405-10644	239
8	*ND4L*	TCCTACTCCCACTTCCCTGA ACGATTGGTATCATGCTGGC	9658-10048	390
9	*ND5*	CCGCAAACAACATATTCCAA AGGCCAAGTAGTGGCAGATT	12196-12405	209
10	*ATP6*	TCATTTACGCCCACAACACA GCAGTAATATTGGCGGTTAATCG	8235-8461	227

Table 3. List of mtDNA changes identified in mitochondrial genes

Gene	Sequence variant	Codon change	Amino acid change	Molecular effect	SIFT for nonsynonymous changes	Frequency (%)
COX1	m.5444T>C	GCT→GCC	p.Ala32=	synonymous	-	39,8
	m.6257G>A	GGC→GGT	p.Ala303=			17,1
COX3	m.8760A>G	TCA→TCG	m.Ser39=			28,4
	m.8764G>T	CTT→CTC	p.Ala41Ser	nonsynonymous	tolerant	27,3
	m.8805A>G	ATA→ATG	p.Met54=	synonymous	-	4,5
	m.8807G>A	TGC→TAC	p.Cys55Tyr	nonsynonymous	tolerant	100
	m.8817A>G	TGA→TGG	p.Trp58=	synonymous	-	13,6
	m.8877A>G	GGA→GGG	p.Gly78=			26,1
ND1	m.3196T>C	CTT→CTC	p.Leu150=			56,8
	m.3254G/A	GAA→AAA	p.Glu170Lys	nonsynonymous	tolerant	36,4
ND2	m.4472A/G	ATA→GTA	p.Met187Val			6,8
	m.4517G>A	GTT→ATT	p.Val202Ile			15,9
ND4L	m.9911_9912insGT	ATG→GTG	p.Met1Val		intolerant	100
ND4	m.10470T>C	ACT→ACC	p.Thr90+	synonymous	-	2,3
	m.10533A>T, m.10533A/T	ACA→ACT	p.Thr111=			21,6 4,5
	m.10542A>G m.10542A/G	GAA→GAG	p.Glu114=			9,1 1,1
	m.10611A>T	GGA→GGT	p.Gly137=			17,0
	m.10613A>G	GGA→GGT	p.Asn138Ser	nonsynonymous	intolerant	3,4
ND5	m.12272T>C	AAC→AAT	p.Asn165=	synonymous	-	11,4
	m.12330A>G	ACC→GCC	p.Thr185Ala	nonsynonymous	tolerant	10,3
	m.12346T>A	CTA→CAA	p.Leu190Gln			3,4
	m.12401T>C	AAT→AAC	p.Asn208=	synonymous	-	2,3
ATP6	m.8281T>C	ATT→ATC	p.Ile106=			18,2

of genes were designed using Primer3web software (version 4.1.0) [Untergasser *et al.* 2012]. The list of designed primers and predicted length of PCR products is presented in Table 2. The amplification of selected fragments of mtDNA was performed with the PCR technique in a Labocycler Thermal Cycler (Sensoquest Biomedical). Then amplification products were visualised using horizontal electrophoresis on 1,5% agarose gel. The amplicons were sequenced using a BigDye Terminator Cycle Sequencing kit (Applied Biosystem, USA) in a 9700 GeneAmp PCR system (Applied Biosystem, USA). The samples were purified using CentriSep columns following the manufacturer's protocol or precipitated with ethanol and sodium acetate following the BigDye kit protocol. Extension products were separated using an ABI 377 automated sequencer (Applied Biosystem, USA).

The obtained mtDNA sequences were compared to the selected fragments from reference sequence (GenBank accession No. U96639) [Kim *et al.* 1998]. Nucleotide sequence changes were analyzed using the Unipro UGENE bioinformatics program (v.34.0) [Okonechnikov *et al*. 2012], followed by protein analysis with bioinformatics tools such as ExPASy Server [Gasteiger *et al*. 2003] and SIFT (Sorting Intolerant From Tolerant) [Combet *et al.* 2000]. The identified DNA and protein variants were described following the guidelines of the Human Genome Variation Society (HGVS) [den Dunnen *et al.* 2016].

Results and discussion

The molecular analyses performed on 88 FFPE samples of malignant MGTs revealed a total 512 nucleotide changes, including mutations in *COX1* (50), *COX3* (176), *ND1* (82), *ND2* (20), *ND4* (56), *ND4L* (88), *ND5* (24) and *ATP6* (16) genes. Additionally, heteroplasmy was observed in *ND1* (m.3254G/A, m.4472A/G), *ND4* (m.10533A/T, m.10542A/G) genes. The most commonly occurred mtDNA variants were m.8807G>A *(COX3)* and m.9911_9912insGT *(ND4L)* followed by m.3196T>C *(ND1)*, m.5444T>C *(COX1)* and m.3254G/A *(ND1)*. The number of mtDNA variants per gene varies from 1 *(ATP6)* to 6 *(COX3)*. The observed mtDNA variants are presented in Table 3.

Changes of mtDNA in the protein-coding genes were mostly synonymous (59,1%). The mutations observed in the samples in positions m.8764G>T, m.8807G>A, m.3254G/A, m.4446T>A, m.4472A/G, m.4517G>A, m.9911_9912insGT, m.10613A>G, m.12330A>G, m.12346T>A caused nonsynonymous changes in the following positions: p.Ala41Ser *(COX3)*, p.Cys55Tyr *(COX3)*, p.Glu170Lys *(ND1)*, p.Ala185Thr *(ND2)*, p.Met187Val *(ND2)*, p.Val202Ile *(ND2)*, p.Met1Val *(ND4L)*. For nonsynonymous mtDNA changes, SIFT analyses were performed. The obtained results indicated that majority of observed nonsynonymous mtDNA variants were tolerant except of m.9911_9912insGT *(ND4L)*, m.10613A>G *(ND4)*.

All samples included in study were malignant MGTs of epithelial origin including: tubulopapillary carcinoma: GI (10), GII (7), GIII (8), complex carcinoma: GI (23),

GII (12), solid carcinoma: GI (1), GII (15), GIII (8), comedocarcinoma: GIII (2) and inflammatory carcinoma: GI (1) and GII (1) - Table 1. The analysis of number of mtDNA variants did not indicate molecular changes specific for histopathological type of MGTs or its grading.

An attempt was made to associate mtDNA changes with individual characteristics based on the available data. The number of molecular changes in each malignant tumour was very diversified and varied from 2 to 13. MGTs were collected from animals aged 3 - 16 years with a predominance of dogs ≥9 years old (82.9%) -Table 1. The comparison between the molecular results and the age indicated high rate of molecular diversity. 27.4% of animals aged ≥9 years old had number of mtDNA changes ≥10. Therefore, the increased age of dogs was not related with higher number of molecular alterations.

Different breeds of dogs are affected with malignant MGTs (Tab. 1). German Shepherds (11/88, 12.5%) with complex carcinomas (4), inflammatory carcinomas (2), and solid carcinomas (5), as well as crossbreed dogs (32/88, 36.4%) with comedocarcinoma (1), complex carcinomas (12), tubulopapillary carcinomas (10), and solid carcinomas (9), prevailed in this study. Detailed analyses of mtDNA changes did not indicate any molecular association between the number or type of mtDNA variants and the development of malignant MGTs in German Shepherds or Crossbreed dogs.

Tumourigenesis is a complex and enormously diversified molecular disorder. Molecular landscape of neoplasms is a result of alternations of DNA and mtDNA. Although various mutations have been reported in well-characterized tumours, the entire process of tumorigenesis remains unknown. [Kozakiewicz *et al.* 2021].

MGTs are the most commonly diagnosed tumours in female dogs with high malignancy rate (>50%) - da Silva *et al.* [2023]. Most research on MGTs has focused on understanding aetiopathogenesis through immuno- and histopathological analyses [Cassali *et al*. 2020, Dolka *et al*. 2016, Muscatello *et al*. 2021, Schulman *et al*. 2022], as well as molecular studies revealing changes in nuclear genes such as BRCA1, BRCA2 [Enginler *et al*. 2014], PIK3CA [Arendt *et al*. 2023], and mitochondrial genes like ATP6, COX1, and ND4L [Kowal *et al*. 2022].

Mitochondria are unique organelles which have their own genetic material. They are crucial in e.g. metabolism of energy, amino acids, fatty acids, steroids and the regulation of the cellular redox status [Kozakiewicz *et al.* 2021]. There are available reports on mtDNA changes in different canine mammary gland tumours [Kowal *et al.* 2022, Slaska *et al.* 2016].

FFPE is a fundamental approach in histopathological diagnosis, as MGT tissue blocks can be used for further analyses, including retrospective molecular research [Di Giacomo *et al*. 2022]. In our study, 88 FFPE tissue blocks were used for retrospective analysis of mtDNA variants in 8 selected mitochondrial genes which frequently undergo changes during tumourigenesis. The results indicate that the quality and quantity of DNA from FFPE materials are sufficient and can be successfully used to sequence DNA from MGTs preserved in formalin-fixed paraffin blocks.

The present analyses showed a diversified landscape of mtDNA of 88 canine MGTs, including 512 mtDNA localised in 23 nucleotide positions including 4 heteroplasmic sites and 1 indel mutation (Tab. 2). It should be noted that two mutations: m.8807G>A *(COX3)* and m.9911_9912insGT *(ND4L)* were present in each analysed malignant sample of MGTs. Other frequently identified mtDNA changes were m.3196T>C *(ND1)*, m.5444T>C *(COX1)* following by heteroplasmy m.3254G/A *(ND1)*. Kowal *et al.* [2022] analysed the whole mitochondrial genome sequencing of blood and tumour tissue samples collected from 13 dogs diagnosed with tubulopapillary carcinomas (6), complex carcinomas (6) and squamous cell carcinoma/carcinoma planoepitheliale keratodes (1). The obtained results indicated 9 commonly identified polymorphisms in each dog, including m.8807G>A *(COX3)* and m.9911_9912insTG *(ND4L)*. Moreover, mtDNA mutation m.8807G>A was also found in Miniature Schnauzer dog with two mammary gland tumours-complex carcinoma (GI) and Dachshund dog diagnosed with comedocarcinoma (GIII) and malignant mixed tumour. In case of *ND4L* gene mutation in form of m.9913_9914insTG was found localised close to m.9911_9912insTG [Kowal *et al.* 2023]. It may imply that indicated nucleotide positions of *COX3* and *ND4L* genes undergo more frequently changes in MGTs. Therefore, these mitochondrial genes could be selected for use in screening diagnostics of neoplasms of canine mammary glands.

Also, other mtDNA changes identified in this study such as m.3196T>C *(ND1)*, m.4517G>A *(ND2)*, m.5444T>C *(COX1)*, m.8281T>C *(ATP6)* were described by other authors [Kowal *et al.* 2023, Surdyka and Slaska 2017]. However, in this case, these examples are rare mutations in comparison to m.8807G>A and m.9911_9912insTG which are described in canine malignant mammary gland tumours.

The risk of MGTs development increases with age. The age of the female dogs at the time of diagnosis was between 9 and 12 years at the time of diagnosis with average age 10.35 years [da Silva *et al.* 2023]. These results are similar to our study where mean age of bitches affected with MGTs was 10.8 years. The majority of dogs with MGTs in our study were crossbreed dogs (36,4%) followed by German Shepherds (12,5%) which are consider as more commonly affected with MGTs [Patel *et al.* 2019, Slaska *et al.* 2016].

Conclusions

FFPE blocks can be used successfully in analysis of mitochondrial DNA alternations. The performed research revealed mtDNA substitutions in *COX1, COX3, ND1, ND2, ND4, ND5, ATP6* genes and an insertion in *ND4L* gene in dogs with MGTs. Variants m.8807G>A *(COX3)* and m.9911_9912insGT *(ND4L)* occurred in all MGTs samples. The commonness of these two mtDNA changes implies that these variants may be selected for molecular diagnostics of MGTs. The observed variability of mtDNA changes across samples may be linked to genomic instability during tumorigenesis. The analysis of mtDNA variants identified in the protein-coding

genes revealed mostly synonymous changes, with nonsynonymous alterations being primarily tolerant and not affecting protein properties.

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