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MOLECULAR DIFFERENCES IN MITOCHONDRIAL GENOMES (MITOGENOMES) OF DOGS WITH RECURRENT AND MULTIPLE TUMOURS AND THEIR REFERENCE TO THE HUMAN MITOCHONDRIAL GENOME*

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Abstract

The aim of this study was to identify molecular defects caused by mutations in mitochondrial DNA in cases of recurrent and multiple canine tumours. We presented molecular differences in the mtDNA genome for two tumours observed in different body parts of five dogs and throughout time in the case of the recurrence. Mitochondrial DNA was sequenced on an Illumina MiSeq sequencer using a 600-cycle kit in a paired-end mode targeting at least 100x coverage. The sequences obtained were subjected to bioinformatic analyses in order to determine mutation and polymorphic sites within the analysed mtDNA genome in the tumour tissue. The total amount of changes: single nucleotide polymorphisms (SNPs), indels, mutations, and heteroplasmy detected in this study was 329. Ten polymorphisms were found in all analysed samples: ins.2679_2680G (*tRNA-Leu* (*UUR*)), m.5367C>T (*COX1*), m.5444T>C (*COX1*), m.6065A>G (*COX1*), m.8368C>T (*ATP6*), m.8807G>A (*COX3*), ins.9913_9914TG (*ND4L*), m.13299T>A (*ND5*), m.15814C>T, and m.16418A>G (control region). Interestingly, the highest number of differences in the mtDNA genome was observed between non-cancerous *pyogranuloma* tissue and *epithelioma glandulae sebacei*. The mutations in the non-cancerous tissue were mainly found in positions where polymorphisms were observed in blood and tumour tissue. The lowest number of changes was observed for the youngest analysed dog, which may indicate that some changes appeared in the mitogenomes with age. There were fewer heteroplasmic alterations in the larger than smaller tumour, which may suggest that the tumour growth is enhanced by genomic instability. The changes in the protein-coding genes were mostly synonymous, and nonsynonymous changes did not lead to alterations in protein properties. New mutations were observed in the post-recurrence tumours in comparison with the pre-recurrent tissue and blood.

Key words: malignant tumours, dog, mtDNA genome, multiple tumours, SNP

Cancer is a common health problem in different breeds of dogs and crossbred dogs. The aetiology of the majority of cancers is multifactorial; hence, it is difficult to indicate specific molecular alterations that are directly related to disease development (Dobson, 2013). Moreover, cancers are a group of diseases resulting from genome instability. Uncontrolled abnormal cell growth causes changes in the energy metabolism of cells. So far, analyses of mitochondria conducted during carcinogenesis have confirmed their involvement in the development of the disease due to an increased level of ROS (reactive oxygen species), hypoxia, and changes in apoptosis signals (Kozakiewicz et al., 2021). The molecular alterations occurring during the development, growth, and tumorigenesis of cancers have frequently been presented in the case of the nuclear genome; however, the literature shows that the mitochondrial DNA also plays an important role in cancer genetics (Kozakiewicz et al., 2021; Ślaska et al., 2016; Tkaczyk-Wlizło et al., 2022). It is worth noting that most studies on animal mitochondrial oncogenetics were based mainly on the analysis of the non-coding region called displacement loop (D-loop) (Ślaska et al., 2014; Surdyka and Slaska, 2017 a) or particular mtDNA genes (Grzybowska-Szatkowska et al., 2014; Slaska et al., 2015).

Our previous mitochondrial genomic analyses were mainly focused on the presence of specific mutations, polymorphisms, and heteroplasmy in malignant mammary tumours (Kowal et al., 2022, 2019). We observed differences between the mtDNA haplotypes of two mammary malignant tumours present in the same dog. Moreo-

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ver, heteroplasmy is often noted in different types of tumours (52%). In some cases, only the heteroplasmic state is observed (Slaska et al., 2015; Śmiech et al., 2019), but in other cases, heteroplasmy is converted into mutations (Śmiech et al., 2016) or mutations and heteroplasmy co-occur (Kowal et al., 2019). The observed mutations depending on the tumour type are mainly substitutions in the *CYTB* or *ND1*, *ND2*, and *ND4* genes (Kowal et al., 2019; Ślaska et al., 2020; Śmiech et al., 2016) and the D-loop with sporadic occurrence of deletions (Bertagnolli et al., 2009). There is no association between the number of mutations or the heteroplasmy level and animal age, tumour grade, and dog origin or breed. In our previous studies of changes in mammary tumours, a commonly occurring defect of mtDNA was homoplasmic mutation m.15814C>T (100%), which was found in mesenchymal, epithelial, and adherent normal mammary gland tissue. Also, position m.15955 was frequently found to undergo mutation m.15955C>T or was heteroplasmic m.15955C/T (58%) in the tested tumour samples. Interestingly, single deletions in the D-loop: m.15931delA and m.15938delG were observed only in three samples (Bertagnolli et al., 2009). On the other hand, Śmiech et al. (2019) analysed mast cell tumours found in different dog breeds and indicated occurrence of heteroplasmy in particular localisations: m.14780C/T and m.14634C/T. Several studies have reported a relatively high occurrence of second malignancies in dogs with endocrine, hepatocellular, and intracranial neoplasms (Rebhun and Thamm, 2010). Yet, there is no information about changes occurring in the mitochondrial genome in different cancer tissues.

The aim of this study was to identify molecular defects caused by mutations in mitochondrial DNA with the use of large-scale genome analysis in cases of recurrent and multiple canine tumours. The comparison of molecular alterations observed in different localizations or pre- and post- recurrence facilitated determination of the plausible role of mitochondrial DNA in the carcinogenesis process. In addition, a comprehensive bioinformatic analysis of the deleterious effects of molecular alterations was performed. As dogs are suitable model organisms for human cancer diseases, especially in the case of mammary gland tumours (Ślaska et al., 2013; Switonski et al., 2004), we indicated that the positions of SNPs corresponded to the human SNPs in the mitochondrial DNA.

Material and methods

Five dogs with various multiple neoplasms were selected for the analysis in order to present the differences at the molecular level. In the first dog, molecular changes in non-cancerous tissue (pyogranuloma) and in a malignant neoplasm were compared to determine differences between benign and malignant tumours. Molecular changes in two independent tumours of different embryonic origin in two tissues were compared in the second dog. In the third dog, molecular changes in a recurrent tumour were compared. Molecular differences in two independent tumours of the same embryonic origin in two tissues were compared in the fourth dog. The fifth dog had two tumours of the same type but differing in size. Samples were collected from each of the five dogs diagnosed with different types of tumours (Table 1). All analysed dogs had two tumours or a recurrence. The tumour tissue sample was placed in a sterile container. Blood was sampled into sterile test-glasses with the K_3EDTA anticoagulant. The tumour tissue samples were subjected to histopathology analysis. The samples were fixed in buffered formalin, pH=7.2, processed routinely, embedded in paraffin wax, sectioned into 4-µm thick fragments, and stained with haematoxylin-eosin and toluidine blue. Microscopic classification was performed in accordance with the WHO histological classification (Hendrick, 1998). The malignancy degree of the mammary gland tumours was assessed using the 3-grade scale of malignancy, i.e. a sum of point values assigned to histomorphological traits according to Goldschmidt et al. (2011). In order to distinguish differences in the ageing rate of breeds of different sizes, the actual dog's age was counted as comparable human age according to the methodology presented in Wang et al. (2020). The size of the dogs was determined according to the animal's body weight and according to the Animal Kennel Club breed standards.

We analysed 16 entire mitochondrial genomes: six genomes obtained from blood, nine genomes from tumour tissues, and one genome obtained from a non-cancerous histopathological alteration – *pyogranuloma*. Each dog had two tumours at the same time or a recurrence that appeared after surgical removal of a tumour. DNA was isolated from post-operative tumour tissues and blood samples from dogs with diagnosed different types of tumours. DNA extraction from blood and tumour tissue was performed with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). DNA samples were assessed quantitatively and qualitatively by electrophoretic separation in 1% agarose gel and spectrophotometrically (Nanodrop DeNovix DS-11, Thermo Fisher, Waltham, USA). Selective amplification of mtDNA was performed on the total genomic DNA using two pairs of primers (Imes et al., 2012). Full coverage of mtDNA was obtained after amplification of two long-range PCR products. PCR reactions were conducted using KAPA HiFi PCR Kit reagents (KAPA Biosystems, Wilmington, USA). The PCR products were analysed subsequently in 0.7% agarose gel and purified using Ampure XP magnetic beads (Beckman Coulter, Brea, USA). DNA quantification analysis was carried out using a Qubit 3.0 fluorimeter (Thermo Fisher Scientific, Waltham, USA). Both PCR products were diluted to the desired concentration and pooled in an equimolar ratio. Such a DNA mixture was then used in library preparation.

Table 1. Information about the dog's metrics (age, size, type of tumour, localization)

Dog						Tumour		
Sample number	Breed	Sex	Size	Metrical age	Comparable human age according to Wang et al. (2020)	Tumour type	Origin	Localization
B15	Mastiff	M	XL	8	64	*pyogranuloma**	mesenchymal	pelvic bone
				epithelioma glandulae sebacei		epithelial	eyelid	
B101	Dachshund	F	M	11	65	*carcinoma in tumor mixtus benignum*	mixed	mammary gland
				carcinoma comedo G3 Ø 2 cm		epithelial	armpit	
B098	Labrador Retriever	M	L	12	77	*tumor mixtus malignum*	mixed	parotid
B119				*tumor mixtus malignum* recurrence		mixed	parotid	
B116	Miniature Schnauzer	F	S	6	40	*carcinoma complexus G1* Ø 5x8 cm	epithelial	mammary gland
				carcinoma complexus G1 Ø 1 cm		epithelial	mammary gland	
B139	Giant Schnauzer	M	L	14	88	*sertolioma*	epithelial	testicle
				epithelioma glandulae perianales		epithelial	anus	

*Histopathological alteration not considered as tumour.

Approximately 1 ng of the PCR DNA template mix was used as an input, and an Illumina shotgun library was constructed using the Nextera XT Kit (Illumina, San Diego, USA) following manufacturer's instructions. The library sample was sequenced on an Illumina MiSeq sequencer using a 600-cycle kit (v3) in a paired-end mode targeting at least 100x coverage. The detailed information about sequencing data generated on the Illumina MiSeq sequencer is presented in Supplementary Table 9. The sequence reads were filtered by quality, and remaining adaptors were removed using the fastp tool (Chen et al., 2018). Cleaned sequencing reads were mapped to the dog mtDNA reference sequence (GenBank accession number: U96639.2) using the bwa aligner (Li, 2013), and consensus generation for each sample was done using samtools and bcftools (Danecek et al., 2021). Sequencing coverage statistics were calculated using the mosdepth tool (https://github.com/brentp/mosdepth).

The nucleotide sequences obtained were subjected to bioinformatic analyses in order to determine mutation and polymorphic sites within the analysed mtDNA genome in the tumour tissue (Unipro UGENE (v. 34.0)) (Okonechnikov et al., 2012). The differences in the nucleotide sequences were determined by comparison thereof with the reference sequence (Kim et al., 1998). The polymorphisms included changes occurring in tumours and blood cells from the same dog in comparison with the reference sequence. A mutation was identified as a change characteristic for mtDNA from one tumour tissue but not present in mtDNA from the blood sample. The determination of reverse mutations was based on the situation when the variants in the reference sequence and one of the tumour sequences were the same, yet different from blood and other tumour tissue polymorphisms. Heteroplasmy was established when one of the variants in a position surpassed 25%.

The bioinformatic analyses were performed with the use of various tools. The ExPASy Server (Gasteiger et al., 2005) was used to characterise such physicochemical parameters as the theoretical isoelectric point, instability index, aliphatic index, and grand average hydropathy. SOPMA was used for calculation of the secondary structural features of antioxidant protein sequences. The structure of tRNA molecules was predicted in the tRNAscan-SE Search Server (Lowe and Chan, 2016). The conservation grade of changes observed in nonsynonymous variants of protein-coding sequences was determined with the use of the ConSurf tool (Ashkenazy et al., 2016). The corresponding positions in human mitochondrial DNA were determined with the Canis mitoSNP tool (Kowal et al., 2023). The alignment of human and canine reference sequences for all genes separately was performed with the use of the Unipro uGene tool, CLUSTALW algorithm. If the human and canine positions matched, they were perceived as corresponding and identical.

The HGVS (2016) nomenclature was used for description of variants of sequences found in the DNA and proteins (den Dunnen et al., 2016). Heteroplasmy was indicated with (†) in each table in supplementary materials.

Results

The molecular changes in mtDNA were detected in twelve out of thirteen protein-coding genes (except

ATP8) of mtDNA, both in rRNA genes and in seven out of 22 tRNA genes (Supplementary Table 1). The total amount of changes (SNPs, indels, mutations, and heteroplasmy) detected in this study was 329. The highest number was represented by polymorphisms – 206 (63%) and reverse mutations – 30 (9%). The vast majority of changes were detected in the VNTR region of the D-loop (108/329), D-loop (40/329), *COX1* (28/329), and *ND5* (22/329). Only two genes encoded on the light strand were found to be altered in comparison to the reference sequence (*ND6, tRNA-Pro*). The heteroplasmy was mainly observed in the VNTR region; however, heteroplasmic mutations were observed in 12s rRNA, *ND4, ND4L*, and *ND6* as well. The indels were mainly observed in *ND4L* and *tRNA-Leu* (*UUR*).

The bioinformatic analysis of the protein-coding sequences revealed 102 changes, from which 30 (29%) were nonsynonymous (Supplementary Table 2). The highest number of nonsynonymous changes was observed in the *ND5* gene, whereas no nonsynonymous variants were detected in the *ATP6* and *CYB* genes. The highest number of synonymous changes (13) was observed in *COX1*. The synonymous shifts were mainly observed for glycine (16/72) and leucine (15/72). It is worth noting that the most frequently observed nonsynonymous changes were shifts from asparagine to serine (4/32), isoleucine to valine (3/32), methionine to threonine (3/32), or leucine to phenylalanine (3/32) (Supplementary Table 2). The codon change was mainly observed in the third position (66/102). The sum of the amino acid shifts was 21 in the most conserved positions and 20 in the most variable positions according to the results provided by the ConSurf tool. Most of the amino acid shifts (68/102) took place in buried positions in the protein structure. Changes in functional residues were detected in the following genes: *ND3* (m.9838G>A → p.Glu115Lys), *ND4* (m.10613A>G → p.Asn138Ser), *ND4L* (ins.9913_9914TG → p.Met1Val, m.9912T>C → p.Met1Thr), and *ND6* (m.14033G/A → p.Pro26=/Leu). The highest number of nonsynonymous shifts involved changes in the alpha helix (12/32) and random coil (15/32) secondary structure. These changes were mainly observed outside (11/30) or in the transmembrane (12/30) region of the proteins. All changes in the β-turn secondary structure were synonymous. Among the protein-coding sequences, 41 out of 102 positions had an identical match to human mitochondrial DNA positions (Supplementary Table 2).

Molecular changes in tRNA-coding genes were observed for *tRNA-Arg, tRNA-Leu* (*UUR*)*, tRNA-Phe, tRNA-Pro, tRNA-Ser* (*AGY*)*, tRNA-Thr*, and *tRNA-Trp*. The alterations were observed in the TΨC loop (3/9) and, consequently, in the central loop, the DHU arm, and the DHU loop (2/9). More changes were observed in 16s rRNA than in 12s rRNA. The alterations in the thermodynamical rRNA secondary structure spanned unpaired bases (8/13) or bases paired upstream 3' (4/13). The vast majority of the molecular changes observed in RNA genes were transitions (17/22). Eleven out of 22 changes in RNA-coding genes corresponded to human mtDNA positions in corresponding genes (Supplementary Table 3). The polymorphisms were found in the following positions in all analysed samples: ins.2679_2680G (*tRNA-Leu* (*UUR*)), m.5367C>T (*COX1*), m.5444T>C (*COX1*), m.6065A>G (*COX1*), m.8368C>T (*ATP6*), m.8807G>A (*COX3*), ins.9913_9914TG (*ND4L*), m.13299T>A (*ND5*), m.15814C>T (control region), and m.16418A>G (control region). Three of them led to a nonsynonymous change in the amino acid sequence: p.Cys55Tyr (COX3), p.Met1Val (ND4L), and p.Ser508Thr (ND5). The largest differences in the number of mutations were found in the non-cancerous and cancerous tissue (dog B15). The lowest number of alterations was observed in the case of a Miniature Schnauzer (B116), which was the youngest and smallest dog of all analysed individuals.

There were overall 73 alterations in the dog (B15) with non-cancerous *pyogranuloma* and malignant *epithelioma glandulae sebacei* tumours, from which 25 were mutations. The highest number of mutations (23/73) was detected in the tumour *epithelioma glandulae sebacei* sample. Among these 23 mutations, 22 were reverse mutations. In contrast, only two mutations (one transition and one heteroplasmic mutation) were observed in the non-cancerous sample. The mutations observed in the sample obtained from *epithelioma glandulae sebacei* (B15G2) in positions m.4572T>C, m.7923T>C, m.9838G>A, m.10346C>T, m.10613A>G, and m.12063G>A caused nonsynonymous changes in the following positions: p.Met220Thr (ND2)*,* p.Ser41Pro (ATP8), p.Glu115Lys (ND3) p.Thr49Met (ND4), p.Asn138Ser (ND4), and p.Val96Ile (ND5). Among these changes, m.9838G>A, m.10613A>G, and m.12063G>A are considered to be placed in conservative regions of the protein, and the first two alterations affected the functional residue (Supplementary Tables 2 and 4).

We identified 51 alterations in the samples obtained from the dog (B101) with two different malignant tumours with different embryonic origins observed at the same time in different tissues. Eight of these 51 changes were mutations. Interestingly, three out of eight mutations were detected in the blood sample. All blood mutations were found in the variable number of tandem repeats (VNTR) region. Five mutations observed in the sample obtained from *carcinoma in tumor mixtus benignum* localised in the mammary gland were mainly heteroplasmy (4/5). One of the variants – m.11482C/T – was found in the *ND4* gene. This heteroplasmic alteration led to a probable nonsynonymous heteroplasmy in the protein structure – p.Pro428=/Ser. This residue was localised in the exposed part of the random coil secondary structure inside the mitochondrial membrane. According to data obtained with the use of the ConSurf tool, the variation in this protein position was likely variable (conservation grade = 4). The canine position m.11482C (*ND4* gene position = 1282) corresponded to the human position m.12041C in the same position in the *ND4* gene. Among 43 polymorphisms observed in these

samples, twelve were identified only in samples collected from this dog: m.2833C>T (*ND1*), m.3676C>T (*ND1*), m.6302A>G (*COX1*), m.8242G>A (*ATP6*), m.8850A>G (*COX3*), m.9896T>C (*tRNA-Arg*), m.11657C>A (*tRNA-Ser* (*AGY*)), m.11813A>G *(ND5*), m.11907T>C (*ND5*), m.15632C>T (control region), m.16025T>C (control region), and m.16198G/A (control region). Most of the polymorphisms in the protein-coding genes were synonymous, except m.11907T>C, which caused nonsynonymous change p.Phe44Leu in the ND5 protein. This position corresponded to human position m.12466T in the same gene (Supplementary Tables 2 and 5). Among the samples obtained from this dog, only the sample of tumour *carcinoma comedo G3* localised in the armpit was not mutated.

The analysis of molecular changes in the dog (B98, B119) with *tumor mixtus malignum* and its recurrence in the parotid gland revealed 49 alterations from which four mutations were observed only in the recurrent tumour sample. These four mutations were observed in positions m.561C/T (12s rRNA), m.10014G/A (*ND4L*), m.16248A (VNTR), and m.16268A (VNTR). The mutations in the VNTR region were identified as reverse mutations, as they were the same as the variants in the reference sequence; yet they differed from the heteroplasmic variants observed in the other samples from this dog. Among the polymorphisms, the variants observed only in the samples of this dog were located in the following positions: m.9041A>G (*COX3*), m.9912T>C (*ND4L*), m.16338G/A (VNTR), and m.16356G/A (VNTR). Interestingly, the polymorphisms in the protein-coding genes were nonsynonymous and caused corresponding changes in the protein structure: p.Asn133Ser (COX3) and p.Met1Thr (ND4L). Both changes affected the random coil secondary structure of proteins localised outside the membrane. The p.Asn133Ser variant was identified as a change in the buried structural residue of the protein, whereas p.Met1Thr was identified as an exposed functional residue of the protein. Both amino acids in these positions were identified as conserved. Both genomic variants: m.9041A and m.9912T corresponded to human positions m.9604A and m.10471T. There were no molecular differences between pre- and post-recurrent blood samples and between blood and pre-recurrent tumour. All four mutations in the post-recurrent tumour tissue were heteroplasmic. The mutation identified in the 12s rRNA gene caused an alteration in the region of unpaired bases in the secondary structure of this RNA molecule. The heteroplasmy in the *ND4L* gene did not cause a nonsynonymous change in the protein (p.Gly35=) (Supplementary Tables 2, 3, and 6).

In the case of the dog with two *carcinoma complexus G1* tumours localised on mammary glands differing in the tumour size, 36 molecular alterations were found. Among these 36 changes, five mutations (m.16148A, m.16178A>G, m.16208A>G, m.16248A>G, m.16298A/G) were found in the VNTR region of the sequence obtained from the bigger tumour (B116G1), and five mutations (m.16158A/G, m.16168A/G, m.16188G/A, m.16218G/A, m.16288A) were found in the VNTR region of the sequence obtained from the smaller tumour (B116G2); interestingly, one mutation (m.16318A/G) in the VNTR region was observed in the blood sample. There were no mutations in the coding regions of the mitochondrial genome. Among all eleven mutations, there were six heteroplasmic mutations, three transitions, and two reverse mutations. There were no mutations in the protein-coding or RNA-coding genes. Three polymorphisms: m.7308A>G (*COX2*), m.10204C>T (*ND4L*), and m.16507T>A (control region) were identified uniquely in the samples of this dog. Consequently, the changes in the protein-coding genes caused a nonsynonymous alteration in the COX2 protein (p.Asn92Ser) and a synonymous alteration in the ND4L gene (p.Cys98=). The nonsynonymous change was localised in the exposed random coil secondary structure outside the mitochondrial membrane; however, the amino acid variability for this residue was graded as high. The synonymous alteration in the ND4L protein was localised in the exposed α-helix secondary structure in the interior part of the mitochondrial membrane, and the conservation grade of this amino acid was high. Both positions m.7308A and m.10204C of these polymorphisms had their identical corresponding position in human mtDNA in positions m.7860A and m.10763C (Supplementary Tables 2 and 7).

The highest number of molecular alterations was found in the dog (B139) with two different tumours of the same epithelial origin in two different tissues. In total, there were 120 changes, with 100 polymorphisms. Among the polymorphisms, 31 were unique only for the samples of this dog. Two unique polymorphisms: m.381T>A and m.557A>G were found in the 12s rRNA gene, whereas six unique polymorphisms: m.1204T>C, m.1454G>A, m.1709G>A, m.1748T>C, m.1756C>T, and m.2232A>G and one mutation: ins.1493_1494A were detected in the 16s rRNA gene. These changes led mostly to alterations in the regions of unpaired bases of the secondary structures or in the region of bases paired upstream 3'. In the tRNA-coding genes, unique polymorphisms were found in the following positions: m.16T>C (*tRNA-Phe*), m.5009C>T (*tRNA-Trp*), ins.9865_9866A (*tRNA-Arg*), m.15372G>A (*tRNA-Thr*), and m.15435G>A (*tRNA-Pro*). Polymorphisms in the tRNA genes led to changes in the DHU loop, the TΨC loop, the DHU arm, or the central loop of the secondary structure (Supplementary Table 3). Among the protein-coding genes, the following polymorphisms caused nonsynonymous changes in the amino acid protein sequence: m.3494T>C (p.Phe250Leu – ND1), m.4503A>G (p.Asn197Ser – ND2), m.4517G>A (p.Val202Ile – ND2), m.6711T>A (p.Ser455Thr – COX1), m.8764G>T (p.Ala41Ser – COX3), m.11402T>C (p.Ile401Thr – ND4), m.11572A>C (p.Ile458Leu – ND4), m.11959C>T (p.Thr61Ile – ND5), m.12330A>G (p.Thr185Ala – ND5), m.12346T>A (p.Leu190Gln – ND5), m.12636T>C

(p.Phe287Leu – ND5), m.12813G>A (p.Val346Ile – ND5), m.13261C>T (p.Thr495Ile – ND5), and m.13791T>C (p.Ile107Gln – ND6). All changes except p.Ile458Leu were identified in the buried structures of the protein. Most of them were found in the transmembrane helix of the mitochondrion. Four of the twelve mutations identified in the samples from this dog were found in *sertolioma* (B139G1) tumour, six in epithelioma *glandulae perianales* (B139G2), and two mutations in the blood sample. Changes in the B139G1 and B139K samples were mainly heteroplasmy in the VNTR region (5/6). There was one mutation (m.634C/T) in the 12s rRNA gene in the testicle tumour. In the anus tumour sample, four out of six mutations were detected in the VNTR region, one in the 16s rRNA gene (m.1493_1494), and the other in the *ND6* gene (m.14033G/A). The heteroplasmic mutation in the *ND6* gene led to a heteroplasmy in the amino acid sequence of the ND6 protein (p.Pro26=/Leu). This nonsynonymous alteration was found in the exposed random coil secondary structure in a functional conserved residue localised outside the mitochondrial membrane. Position m.14033G corresponded to human position m.14600C (Supplementary Tables 2, 3, and 8).

Discussion

Dogs have earned the status of family members mainly in Western societies, but also in East Asian societies like Japan (Ambros, 2015). Dog owners now expect more advanced veterinary healthcare and investment in more resources for diagnosis technologies and pioneer therapeutics like those applied to humans. Analyses of changes occurring in dog's mitogenomes may help to understand the molecular aspects of carcinogenesis, and the identification of mutations and polymorphisms frequently observed in dogs with tumours may be used in molecular diagnostics. In recent years, tumour biomarkers, such as p53, Bcl-2 (B-cell lymphoma-2), Bax, and COX-2 (cyclooxygenase-2), have been widely used for early diagnosis, monitoring, and determining the prognosis of canine malignant tumours (Anadol et al., 2017). In this study, ten polymorphisms were found in all analysed samples. The following polymorphisms: ins.2679_2680G (*tRNA-Leu* (*UUR*)), m.5367C>T (*COX1*), m.5444T>C (*COX1*), m.6065A>G (*COX1*), m.8368C>T (*ATP6*), m.8807G>A (*COX3*), ins.9913_9914TG (*ND4L*), m.13299T>A (*ND5*), and m.15814C>T (control region) were already observed in our previous articles (Kowal et al., 2022, 2019; Slaska et al., 2015; Surdyka and Slaska, 2017 b). However, it should be emphasised that the actual effect of these changes remains unclear.

There has been an increase in the proportion of patients suffering from multiple mammary gland tumours from 19.6% in 2003 to 43.0% in 2020, as reported by Rodríguez et al. (2022). One of the most frequent types of multiple tumour described in the literature was canine mast cell tumour (Tamlin et al., 2022). In this research, we presented the results of the whole mitogenome analysis of dogs with recurrent and multiple tumours. To the best of our knowledge, this is the first description of such changes in canine oncology. We described the molecular differences in the mitogenomes observed in dogs with multiple and recurrent tumours. Interestingly, the highest number of differences in the mtDNA genome was observed between non-cancerous *pyogranuloma* tissue and *epithelioma glandulae sebacei*. Noteworthy, the positions of the mutations observed in *epithelioma glandulae sebacei* tumour coincided with the positions of polymorphisms observed in blood and non-cancerous tissue. Therefore, mtDNA mutations may contribute to the cell's potential to become a cancer cell (van Gisbergen et al., 2015). Hypothetically, the single nucleotide polymorphisms observed in the mitogenome might be actually the hotspots of reverse mutations in malignant tumours. Genetic alterations resulting in a dysfunctional electron transport chain generate excessive levels of mitochondrial reactive oxygen species (mtROS) (Lagouge and Larsson, 2013). In physiological conditions and during the early stages of disease, mitochondria produce moderate levels of mtROS that are beneficial to cellular growth and survival. However, as the mitochondrial dysfunction worsens, the levels of mtROS can exceed the tolerable threshold and become lethal to tumour cells (Galadari et al., 2017; Inigo and Chandra, 2022). The increased mitochondrial activity required by cancer cells leads to increased production of mtROS. This results in elevated levels of oxidative damage and mtDNA mutations, leading to mitochondrial dysfunction. A vicious cycle develops, in which the mitochondrial dysfunction aggravates the mtROS generation, leading to further mitochondrial dysfunction (Inigo and Chandra, 2022). Supposedly, the most prone regions to mtROS damage are in fact polymorphic sites of the mitochondrial genome that were already altered in the past. Yet, it cannot be excluded that many changes in mtDNA caused too much damage to cancer cells that have been eradicated; therefore, no significant mutations are observed in tumour mitogenomes in other analysed malignant tumours. Gilkerson et al. (2012) showed that defective mitochondria displayed increased mitophagy (Gilkerson et al., 2012). When a mutation leads to a reduced mitochondrial membrane potential (depolarization of mitochondria), the mutation causes a phenotypical change and therefore mitochondria lose their ability to function normally. Consequently, the mitochondria are not able to re-fuse with the mitochondrial network after fission and are recycled (Twig and Shirihai, 2011).

The lowest number of differences of all the analysed samples was observed in the youngest dog (B116). Age-related somatic mtDNA mutations accumulate in post-mitotic tissues until a certain tissue-specific threshold in the level of mutant to normal mtDNA molecules is surpassed and cells become energetically compromised (Wallace, 2010). Hence, the main consequence of mtD-

NA mutations is an impairment of energy metabolism, inducing ageing effects on tissues that display high energetic demands, such as the heart, skeletal muscle, and the brain (Zapico and Ubelaker, 2013). The accumulation of mtDNA mutations, including deletions, duplications, and point mutations, has been found in a variety of tissues during ageing in humans, monkeys, and rodents (Zapico and Ubelaker, 2013). The age of the analysed dogs compared to human age was between 40 and 88 years old; therefore, it cannot be excluded that some of the changes may be linked with the ageing process. However, it should be emphasised that the presence of changes caused by the ageing process should be examined separately from damage possibly linked with the carcinogenesis process on a larger cohort of healthy dogs.

Mitochondria contribute to various stages of cancer development and progression despite their dysfunctional state. Mutations in mitochondrial genes may provide an advantage in early tumour growth, as this was observed in the case of mutations of the human *ATP6* gene leading to an increase in superoxide production from complexes I, II, and III (Shidara et al., 2005). The m.8993T>G mutation in human *ATP6* specifically favours tumorigenicity in human prostate cancer, whereas a polymorphism m.8425G>A was observed in canine *sertolioma* tumour, which corresponded to the human m.8988A position. Moreover, mutations in mtDNA, particularly in the ND genes, enhance the metastatic activity of cancer cells (Inigo and Chandra, 2022). However, most of the changes identified in this study had a minor or mild effect on OXPHOS functioning, leading to some changes in the protein structure. Most of the amino acid changes observed in the study did not change the protein properties significantly and were placed in the buried parts of the proteins. Among the fifteen alterations that affected the functional sites in the protein secondary structure, only five: m.9838G>A → p.Glu115Lys (ND3), m.9912T>C → p.Met1Thr (ND4L), ins.9913_9914TG → p.Met1Val (ND4L), m.10613A>G → p.Asn138Ser (ND4), and m.14033G/A → p.Pro26=/Leu (ND6) were nonsynonymous. Similarly, among the twelve alterations in the protein secondary structure, only two: m.9041A>G → p.Asn133Ser (COX3) and m.12813G>A → p.Val346Ile (ND5) were nonsynonymous. Yet, it should be emphasised that the ins.9913_9914TG was observed in all the analysed samples and in the previous reports of changes in canine mitochondrial genomes (Kowal et al., 2022, 2019). Interestingly, genes encoded on the complement strand seemed to be less prone to alterations in the sequence.

Spontaneous tumours in pet dogs have the potential to bridge the gap between preclinical models and human clinical trials. Due to its reasonable cellular, molecular, and genetic homology to humans, the pet dog represents a valuable model accelerating the translation of preclinical studies into clinical trials in humans, actually with benefits for both species (Ślaska et al., 2013; Switonski et al., 2004). Interestingly, clinical human studies are also informative to pet dog cancer research. Therefore, humans may also be a "model" to pet dogs (Klingemann, 2018). Moreover, their unique genetic features of breeding and breed-related mutations have contributed to assessment and optimisation of therapeutics in individuals with different genetic backgrounds (Mestrinho and Santos, 2021). Therefore, in this article, we demonstrated corresponding positions in the human mtDNA genome in order to facilitate the comparison between these two species. Interestingly, some changes, e.g. ins.2679_2680G in the canine genome, corresponded to the m.3239G position in the human genome lying in the *TRNL1/MT-TER* region. Although the presence of the *MT-TER* region has not been confirmed in the canine genome, the possible effect of a change in this region may cause alterations in the mitogenome replication process. However, further analysis is necessary.

The risk of tumour development increases with age, which was confirmed by Grüntzig et al. (2016) in adenoma/adenocarcinoma, melanocytic tumours, and squamous cell carcinoma. The skin and the female reproductive system seem to be the most common locations of cancer in pet dogs (Brønden et al., 2010). Interestingly, the exceptional longevity in dogs seems to have a cancer-resistant phenotype just like in the oldest-old humans (Cooley et al., 2003). Clinically, the tumour size strongly influences the prognosis of canine mammary tumours, i.e. dogs with larger tumours have a poorer remission and survival prognosis than those with smaller tumours (Zheng et al., 2022). Interestingly, we observed that the larger tumour had fewer heteroplasmic mutations than the smaller one, which suggests that the tumour growth may be favoured by a more stable mitogenome, which in turn leads to more effective production of energy necessary for growth. Moreover, the number of heteroplasmic sites observed in the VNTR region was higher in the smaller tumour, which may suggest higher genome instability. However, this hypothesis should be verified on a larger study group.

Microsatellite instability is one of the mechanisms responsible for genomic instability in cancers. Microsatellite instability is linked to mutations in the mismatch repair (MMR) system (Deshpande et al., 2020) and are found in hereditable cancers such as Lynch syndrome (Yao and Dai, 2014). In all the analysed samples, VNTR region instability was observed. Interestingly, variant m.16418A>G being a part of the VNTR region was observed in all the analysed dogs. Heteroplasmic mutations and heteroplasmy were observed in almost all positions of the 300-bp fragment of the control region. VNTRs are an important source of RFLP genetic markers used in linkage analysis (mapping) of genomes. They have become essential in forensic crime investigations. VNTRs are used to study genetic diversity (DNA fingerprinting) and breeding patterns in animals (Marwal et al., 2014). In bacteria, VNTRs have been implicated in some very important biological processes, where they generate genetic variation faster than other genomic regions (Keim,

2013). In humans, VNTRs have been implicated as regulators of both local epigenetics and gene expression levels and as modifiers of disease susceptibility in a variety of conditions, including Alzheimer's or schizophrenia (Garg et al., 2022). Kowal et al. (2020) found a plausible CpG island in the VNTR region in the canine D-loop (Kowal et al., 2020). Although the function of this region in canine mitochondrial DNA remains unclear, it cannot be excluded that the variability in this region may alter the epigenetic motifs of methylation linked with carcinogenesis. On the other hand, the heteroplasmic sites in the repeating motif GTACACGTA/GC may be an effect of the combination of two complex repetitive elements. It is worth noting that changes in this region were observed in blood samples as well, which may suggest that the recombination is not directly related to carcinogenesis.

Conclusions

The molecular analysis of changes in the mitochondrial DNA of dogs with tumours revealed ten polymorphisms in all the analysed samples: ins.2679_2680G (*tRNA-Leu* (*UUR*)), m.5367C>T (*COX1*), m.5444T>C (*COX1*), m.6065A>G (*COX1*), m.8368C>T (*ATP6*), m.8807G>A (*COX3*), ins.9913_9914TG (*ND4L*), m.13299T>A (*ND5*), m.15814C>T, and m.16418A>G (control region). The mitogenome of the non-cancerous tissue was less mutated than the cancerous tissue. Most of the mutations were reverse and were placed in the same positions as polymorphisms in healthy tissue. Therefore, the single nucleotide polymorphisms observed in the mitogenome might be actually the hotspots of reverse mutations in malignant tumours. The number of mutations in tumours differing in size was the same; however, the smaller tumour had more heteroplasmic types of mutations that may be linked with higher genomic instability. The changes in the protein-coding genes were mostly synonymous, and nonsynonymous changes did not lead to alterations in protein properties. The lack of significant deleterious mutations in the mitochondrial genomes may have been caused by their eradication from the cells that were unable to grow and develop with damaged mitochondria. In the post-recurrence tumours, new mutations were observed in comparison with the pre-recurrent tissue and blood. The lowest number of differences was observed in the youngest dog, which suggests that some of the changes may be linked with the ageing process. The alterations in the VNTR region may play a key role in carcinogenesis leading to disturbances in the replication or transcription of mtDNA genes; however, this hypothesis should be thoroughly verified.

Ethical statement

The study was approved by the II Local Ethical Commission for Animal Experiments in Lublin, Poland (Resolution number 6/2013).

Conflict of interest

The authors declare no competing interests.

Supporting information

The data that supports the findings of this study are available in the supplementary material of this article. The data obtained after NGS sequencing generated in this study have been submitted to the NCBI BioProject database under accession number PRJNA881939.

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