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The occurrence of changes in mitochondrial *tRNA-Leu (UUR)* gene and its methylation profil in dogs with malignant tumours*

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The study aimed to identify molecular alterations in the *tRNA-Leu* (*UUR*), compare the methylation profile of this gene in healthy dogs *versus* dogs with malignant tumours, and to analyse whether there is a link between the presence of these changes and tumorigenesis. This study included 74 samples obtained from 47 dogs. The experimental group included 19 individuals with various malignant tumors (blood, tumor tissue) and 8 bitches with carcinoma tubulo-papillare (tumor and healthy tissue), one of the most common cancers in these animals. Notably, this study represents the first-ever analysis of the canine *tRNA-Leu* (UUR) gene methylation profile. The control group comprised 20 individuals without signs of disease (blood) of varying ages and breeds. Sanger sequencing was used, followed by bioinformatic assessments of the obtained sequences. Thus, the assessments revealed two variations: the m.2683A>G polymorphism present in 81% of the samples from the experimental group and in 85% of the samples from control group, as well as the m.2678_2679insG polymorphism, present in 100% of both groups. Furthermore, no changes were detected in the methylation profile of the *tRNA-Leu* (*UUR*) in both tumour and healthy tissue samples from the selected dogs. The detailed study did not show any tendency between observed molecular changes and type of the tumour. Further study is needed to verify the occurrence of alternations to analyse the molecular background of the disease comprehensively.

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The incidence of canine cancers is on the rise and is a significant cause of mortality especially in senior dogs [Vazquez *et al*. 2023]. Regrettably, despite extensive research and a considerable degree of awareness among the owners of these animals, the precise cause of this condition remains unclear. Canine neoplasms, like those of humans, are characterised by a high heterogeneity and are divided into different types and degrees of malignancy i.e. known for their high metastatic potential [Morris 2016]. The tumour development is associated with genome instability. Changes occurring in the nucleotide sequence as well as the structure of the DNA, spreading during the replication process lead to uncontrolled and abnormal cell growth and oncogenic activation [Sarver *et al.* 2022]. Noticeably, there is a clear link between carcinogenesis and the replication process.

Currently, most research focuses mainly on the analyses of the nuclear genome; however, mitochondrial DNA replicates in postmitotic cells throughout the organism's lifetime, regardless of the cell cycle phase [Larsson 2010, Sarver *et al.* 2022]. Previous studies on the involvement of mitochondria in cancer confirmed their involvement in progression due to the increased levels of reactive oxygen species (ROS), hypoxia and changes in apoptosis signalling [Kozakiewicz *et al.* 2021, Tkaczyk-Wlizło *et al.* 2022]. Moreover, an elevated level of mitochondrial oxidative stress can lead to mtDNA demethylation. Although mitochondrial genes are considered housekeeping genes, alterations in the DNA methylation pattern have already been documented in humans and mice [Bellizzi *et al*. 2013]. Current studies on this subject in dogs remain relatively scarce. To date, researchers have described methylation changes in canine tumours, including osteosarcomas, lymphomas, and mammary tumours (intron motifs of the *CDH2*, *ADAM19*, *CDH5*, and *LRIG1* genes) - Bryan *et al.* [2009], Noguchi *et al.* [2015], Bronzini *et al.* [2017], Nam *et al.* [2020] and Montaner-Angoiti *et al.* [2023].

According to data from GenBank, the canine mitochondrial genome is made up of 16,727 bp in total encoding 37 genes, among which 22 tRNA genes considered as "hot spots" for mtDNA mutations [Kim *et al.* 1998]. So far, analyses based on mitochondrial DNA have revealed some alternations associated with tumour development in dogs in genes such as: *COX2, COX3, ND1, ND2, ND4, CYTB*, and D-loop region [Slaska *et al.* 2014, 2015, Surdyka and Slaska 2017ab, Kowal *et al.* 2019, 2022, 2024, Ziółkowska *et al.* 2023]. Based on the available data, tRNA genes have not been a common molecular target in the identification of the canine disease development. However, studies conducted to date suggest that changes in the sequence of the *tRNA-Leu (UUR)* gene m.2683G>A, m.2678_2679insG may be involved in canine tumorigenesis [Kowal *et al.* 2019, 2022, Ziółkowska *et al.* 2023, Kowal *et al.* 2024].

This is the first study to analyse the canine methylation profile of tRNA-Leu (UUR). The study aimed to identify molecular alterations in the *tRNA-Leu* (*UUR*), compare the methylation profile of this gene in healthy dogs *versus* dogs with malignant tumours, and to analyse whether there is a link between the presence of these changes and tumorigenesis.

Material and methods

The biological material consisted of 74 samples obtained from 47 dogs, which were divided into two study groups. The experimental group comprised of 27 subjects of various ages and breeds. It included 19 dogs (D1-D19) diagnosed with carcinoma complexus and haemangiopericytoma, from which tumour tissue and blood samples were collected during routine surgeries, and 8 dogs (D20-D27) diagnosed with tubulo-papillary carcinoma, with samples taken from both tumour and healthy tissues for methylation profile analyses. The control group consisted of 20 dogs without symptoms of disease, of various ages and breeds, from which blood samples were collected (D28-D47). The detailed information on canine samples that were used for the study is shown in Table 1.

All samples were collected in veterinary clinics following Resolution number 79/2014 of the II Local Ethical Committee for Animal Experiments in Lublin, Poland. The tumour tissue samples were carefully placed in a sterile container, while the blood samples were collected into sterile test glasses containing K_3EDTA anticoagulant (Medlab, Raszyn, Poland). Tumour tissues were examined histopathologically to identify their type and grade of malignancy. The tumours 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. Next, the tissues were embedded in paraffin blocks in a tissue processor (Leica TP-1020, Leica Biosystems, Nussloch, Germany). A 4 μm thick tissue sections were prepared with a sledge microtome (Leica SR-200, Leica Biosystems, Nussloch, Germany) and placed on microscope slides. For histopathological evaluation, the preparations were stained with haematoxylin 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 mammary gland tumours was assessed using a 3-grade scale, based on the sum of point values assigned to histomorphological traits according to Goldschmidt *et al*. [2011].

The extraction of DNA from all tissues was conducted with the use of a DNeasy Blood & Tissue Kit from Qiagen. The quality of the DNA samples was assessed through the qualitative analysis of electrophoretic separation in a 1.5% agarose gel. Additionally, the quantitative analysis was performed using the Nanodrop DeNovix DS-11 from Thermo Fisher (Waltham, USA).

The amplification of the *tRNA-Leu (UUR)* gene was performed with the use of polymerase chain reaction (PCR) technique in a Labocycler Thermal Cycler (Sensoquest Biomedical). The temperature-time profile of the reaction and the composition of the reaction mixture for the *tRNA-Leu* (*UUR*) gene were determined experimentally. PCR reactions were conducted using KAPA HiFi PCR Kit reagents (KAPA Biosystems, Wilmington, USA). Primers used in the analyses were the following: LEU1F 5'- CAAGGGATGTAAGGCCTACC3'and LEU1R 5'-TACGGCAAGAAGGATTGGGA3', and were designed by the authors using Primer3 program (v. 0.4.0) [Untergasser *et al.* 2012]. A 231 bp product covered the 2567-2797

Table 1. Information on canine samples used in the study

	Tumour histopathological classification	Tumour location	Grade	Breed	Age (years)	Sex	Laboratory number
Experimental group	*carcinoma complexus*	nipple	G1	Crossbreed	7	F	D1
			G2		14		D5
			G2	Dachshund	15		D2
			G1		14		D4
			G2		10		D8
			G1	German shepherd	12	M	D7
			nd*		8	F	D9
			G2	Pitbull	11		D10
			G2	Spaniel	11		D3
			G2		13		D6
	carcinoma tubulo-papillare		G2	Crossbreed	16		D23
			G3	German Shepherd	8		D21
			G1		8		D22
			G3		12		D24
			G2		5		D25
			G1		7		D27
			G2	German Shepherd Mixed Breed	10		D26
			G1	Great Dane	7		D20
	haemangiopericytoma	elbow region	nd*	Crossbreed	7	M	D11
		flank			10	F	D15
		metatarsal			11		D16
		body side			13	M	D18
		nipple			12	F	D19
		toe		German shepherd	7		D13
		forearm			14	M	D14
		scrotum			11		D17
		tail region		Weimaraner	7	F	D12
Control group	without signs of a disease			American Staffordshire Terrier	5,5	M	D28
					9,5		D29
					10	F	D30
				Bernese Mountain Dog	2	M	D31
					8	F	D32
				Boxer	8		D33
					6	M	D34
					1,5	F	D35
				Crossbreed	7		D41
					7		D42
					7	M	D43
				Dachshund	12	F	D37
				Golden Retriever	7 months	M	D36
				Labrador Retriever	3		D38
					8	F	D39
					8	M	D40
				Schnauzer	5	F	D46
				Shar Pei	6		D44
					4		D45
				Weimarner	7		D47

*Not applicable

region of the canine mitochondrial genome was taken into consideration. The PCR thermocycling program was set to 95°C for 10 min; 35 cycles of 95°C for 45 s, 52°C for 45 s, and 72°C 2 min; 72°C for 5 min s. Then the reactions were subsequently cooled to 4°C. The PCR products were subsequently purified with a combination of two enzymes: shrimp alkaline phosphatase (FastAP™ Thermosensitive Alkaline Phosphatase (SAP, Thermo Fisher Scientific, USA). The Sanger sequencing service was outsourced to an external company.

The selected group consisted of 8 bitches diagnosed with *tubular papillary carcinoma* of the nipple (Tab. 1). For the purpose of conducting analyses, we have designed a pair of primers (Forward: GGATGTAAGGTTTATTTTATAGAGG and Reverse: AAAAAAAATAATATTAATAAAAAACATTTT) for BS-PCR reaction in the MethPrimer tool [Li and Dahiya 2002]. A 203 bp product containing 4 CpG sites and 35 non-CpG cytosines spanned the 2572-2774 region of the canine mitochondrial genome. The specific region was chosen according to the analyses conducted by

Kowal *et al.* [2020]. The isolated DNA from both tissues was converted with the use of EpiTect Fast DNA Bisulfite Kit (QIAGEN). Due to the high degradation after bisulfite conversion, Whole Bisulfiitome Amplification was executed according to the manufacturer's protocol (QIAGEN). The converted and amplified DNA matrix was then used for BS-PCR reaction (PCR reaction mix: 13,4 µl water, 2 µl of 10x PCR buffer, 0,8 µl of 25 mM $MgCl_2$, 1,6 µl of dNTPs, 0,5 µl of each primer diluted 10x, 0,2 µl of HotStar polymerase and 1µl of converted DNA). The touchdown PCR cycling conditions were: 95°C for 10 min, followed by 5 cycles at 97°C for 5 sec, 58°C for 2 min and 72°C for 45 sec, and further 35 cycles at 97°C for 5 sec, 60 or 56°C for 2 min, 72°C for 45 sec and the final elongation at 72°C for 7 min [Semik-Gurgul *et al.* 2018]. To purify BS-PCR products from excess primers and deoxyribonucleotides, a combination of two enzymes: shrimp alkaline phosphatase (FastAP™ Thermosensitive Alkaline Phosphatase, SAP, Thermo Fisher Scientific, USA) and *E. coli* exonuclease I (Exo I, Thermo Fisher Scientific) was used The Sanger sequencing external service was used for sequence generation.

The sequences were analysed in order to indicate the methylation sites and the sites of mutations and polymorphisms in the *tRNA-Leu* (*UUR*) gene using Unipro UGENE tool [Okonechnikov *et al.* 2012]. To compare these sequences, the CLUSTALW algorithm was used. The nucleotide sequences of *tRNA-Leu* (*UUR*) gene in each dog were compared with the reference sequence (GenBank accession No. U96639).

Results and discussion

The performed analyses revealed two polymorphisms: m.2678_2679insG and m.2683G>A in the sequences of the mitochondrial gene *tRNA-Leu (UUR).* A polymorphism was defined as a change occurring in both examined tissues of an individual, compared to the reference sequence. The study did not reveal any additional alterations in the collected biological material, such as mutations or heteroplasmy.

The m.2683A>G polymorphism was observed in 81% of the samples from the experimental group. Its absence was noted in all analysed tissues of three individuals (German Shepherd, Weimaraner, Crossbreed) diagnosed with *haemangiopericytoma*, as well as in a German Shepherd with *carcinoma complexus* and a German Shepherd diagnosed with *carcinoma tubulo-papillare*. It is noteworthy that there was no increased tendency for this alteration to occur in any specific type of tumour. Among a control group, consisting of individuals without symptoms of the disease, the m.2683A>G lesion was observed in 85% of the tested dogs. No changes were observed in the sequences of the Bernese Mountain Dog and two Crossbreed dogs. The m.2678_2679insG polymorphism was observed in 100% of dogs in both the experimental and control groups (Tab. 2). It is noteworthy that the frequency of both lesions was similar across all study groups. Previous research on canine cancer found that the frequency of m.2678_2679insG and m.2683G>A was similar to the results of the present study; however, it did not include sequence analyses

Table 2. The occurrence of changes in mitochondrial *tRNA-Leu (UUR)* gene

Laboratory number	Tumour histopathological classification	seq ref.		Occurrence of the variant tissue		
seq ref. No. U96639				tumour	blood	healthy
D1		m.2683G	m.2678_2679	m.2683G>A m.2678_2679insG	m.2683G>A m.2678_2679insG	
D2		m.2683G	m.2678_2679	m.2683G>A m.2678_2679insG	m.2683G>A m.2678_2679insG	
D3		m.2683G	m.2678_2679	m.2683G>A m.2678_2679insG	m.2683G>A m.2678_2679insG	
D4		m.2683G	m.2678_2679	m.2683G>A m.2678_2679insG	m.2683G>A m.2678_2679insG	
D5	*carcinoma complexus*	m.2683G	m.2678_2679	m.2683G>A m.2678_2679insG	m.2683G>A m.2678_2679insG	
D6		m.2683G	m.2678_2679	m.2683G>A m.2678_2679insG	m.2683G>A m.2678_2679insG	
D7		m.2683G	m.2678_2679	**m.2683G** m.2678_2679insG	**m.2683G** m.2678_2679insG	
D8		m.2683G	m.2678_2679	m.2683G>A m.2678_2679insG	m.2683G>A m.2678_2679insG	
D9		m.2683G	m.2678_2679	m.2683G>A m.2678_2679insG	m.2683G>A m.2678_2679insG	
D10		m.2683G	m.2678_2679	m.2683G>A m.2678_2679insG	m.2683G>A m.2678_2679insG	
D11		m.2683G	m.2678_2679	m.2683G>A m.2678_2679insG	m.2683G>A m.2678_2679insG	
D12		m.2683G	m.2678_2679	**m.2683G** m.2678_2679insG	**m.2683G** m.2678_2679insG	
D13		m.2683G	m.2678_2679	m.2683G>A m.2678_2679insG	m.2683G>A m.2678_2679insG	
D14		m.2683G	m.2678_2679	**m.2683G** m.2678_2679insG	**m.2683G** m.2678_2679insG	
D15	*haemangio-pericytoma*	m.2683G	m.2678_2679	m.2683G>A m.2678_2679insG	m.2683G>A m.2678_2679insG	
D16		m.2683G	m.2678_2679	m.2683G>A m.2678_2679insG	m.2683G>A m.2678_2679insG	
D17		m.2683G	m.2678_2679	m.2683G>A m.2678_2679insG	m.2683G>A m.2678_2679insG	
D18		m.2683G	m.2678_2679	**m.2683G** m.2678_2679insG	**m.2683G** m.2678_2679insG	
D19		m.2683G	m.2678_2679	m.2683G>A m.2678_2679insG	m.2683G>A m.2678_2679insG	
D20		m.2683G	m.2678_2679	m.2683G>A m.2678_2679insG		m.2683G>A m.2678_2679insG
D21		m.2683G	m.2678_2679	**m.2683G** m.2678_2679insG		**m.2683G** m.2678_2679insG
D22		m.2683G	m.2678_2679	m.2683G>A m.2678_2679insG		m.2683G>A m.2678_2679insG
D23	*carcinoma tubulo-papillare*	m.2683G	m.2678_2679	m.2683G>A m.2678_2679insG		m.2683G>A m.2678_2679insG
D24		m.2683G	m.2678_2679	m.2683G>A m.2678_2679insG		m.2683G>A m.2678_2679insG
D25		m.2683G	m.2678_2679	m.2683G>A m.2678_2679insG		m.2683G>A m.2678_2679insG
D26		m.2683G	m.2678_2679	m.2683G>A m.2678_2679insG		m.2683G>A m.2678_2679insG
D27		m.2683G	m.2678_2679	m.2683G>A m.2678_2679insG		m.2683G>A m.2678_2679insG
D28		m.2683G	m.2678_2679		m.2683G>A m.2678_2679insG	
D29		m.2683G	m.2678_2679		m.2683G>A m.2678_2679insG	
D30		m.2683G	m.2678_2679		m.2683G>A m.2678_2679insG	
D31		m.2683G	m.2678_2679		**m.2683G** m.2678_2679insG	
D32		m.2683G	m.2678_2679		m.2683G>A m.2678_2679insG	
D33		m.2683G	m.2678_2679		m.2683G>A m.2678_2679insG	
D34		m.2683G	m.2678_2679		m.2683G>A m.2678_2679insG	
D35		m.2683G	m.2678_2679		m.2683G>A m.2678_2679insG	
D36		m.2683G	m.2678_2679		m.2683G>A m.2678_2679insG	
D37	without signs of disease	m.2683G	m.2678_2679		m.2683G>A m.2678_2679insG	
D38		m.2683G	m.2678_2679		m.2683G>A m.2678_2679insG	
D39		m.2683G	m.2678_2679		m.2683G>A m.2678_2679insG	
D40		m.2683G	m.2678_2679		m.2683G>A m.2678_2679insG	
D41		m.2683G	m.2678_2679		**m.2683G** m.2678_2679insG	
D42		m.2683G	m.2678_2679		**m.2683G** m.2678_2679insG	
D43		m.2683G	m.2678_2679		m.2683G>A m.2678_2679insG	
D44		m.2683G	m.2678_2679		m.2683G>A m.2678_2679insG	
D45		m.2683G	m.2678_2679		m.2683G>A m.2678_2679insG	
D46		m.2683G	m.2678_2679		m.2683G>A m.2678_2679insG	
D47		m.2683G	m.2678_2679		m.2683G>A m.2678_2679insG	

of healthy dogs. Nevertheless, it should be noted that these alterations were also present in tumour types not included in the present study, such as *carcinoma planoepitheliale keratodes* and *carcinoma solidum* [Kowal *et al.* 2019, 2022, Ziółkowska *et al.* 2023].

The analysis of mitochondrial DNA identified mutations and polymorphisms associated with disease development. To date, in cases of canine malignant mammary gland tumours, mutations in mitochondrial genes such as COX2, COX3, ND1, ND2, ND4, CYTB, and the D-loop region have been reported [Bertagnolli *et al*. 2009, Stewart *et al*. 2015, Kowal *et al*. 2019, Tkaczyk-Wlizło *et al*. 2022, Ziółkowska *et al.* 2023]. It has been established that the D-loop is the mtDNA region with the highest number of mutations and polymorphisms [Slaska *et al.* 2014, Kowal *et al.* 2019]. On the other hand, it has been determined that more than a half of disease-causing mtDNA mutations occur in tRNA genes, which constitute approximately only 9% of the mtDNA [Baranowska *et al.* 2009].

It is important to note that the m.2683G>A dog`s polymorphism overlaps with the human m.3243 position [Kowal *et al.* 2022]. Moreover, the m.3243A>G mutation is recognized as a deleterious change, occurring in most individuals with MELAS syndrome [Finsterer 2007]. A study performed by Kowal et al. [2022]which in turn may provoke similar deleterious effects. The homology between the human MT-TL1 and canine *tRNA-Leu* (UUR showed that the m.2683G>A alternation did not change the secondary structure of canine *tRNA-Leu* (*UUR*), while the corresponding human m.3243A>G seemed to result in a more unstable form of the *tRNA-Leu* (*UUR*) molecule. Due to its structure, mitochondrial DNA undergoes changes much more frequently than nuclear DNA. Persistent nucleotide sequence alterations can lead to haplotype differentiation, with some linked to increased susceptibility to human diseases, such as European haplogroup N and a higher risk of breast tumours [Grzybowska-Szatkowska and Slaska 2012ab]. It is possible that the polymorphisms detected in this study are characteristic for the Polish dog population. It has been suggested that the presence of polymorphisms influences the increased production of free radicals [Grzybowska-Szatkowska and Slaska 2012b]. Therefore, indicated polymorphisms may be involved in the process of carcinogenesis in in conjunction with accompanying mutations.

Epigenetics, and consequently the study of gene methylation (particularly mitochondrial DNA), is still a young field of science. Nevertheless, it is believed that modifications in tRNA methylation can disrupt the synthesis of the respiratory chain, leading to an increase in mitochondrial energy metabolism levels [Yuan *et al.* 2024]. In the present study, among a selected group of eight individuals diagnosed with tubular papillary carcinoma, no changes in the methylation profile of the *tRNA-Leu* (*UUR*) gene were found for both cancer and healthy tissue samples. The DNA methylation is one of the key epigenetic markers involved in the regulation of gene expression, now increasingly used in the study of many diseases. Differences in methylation patterns have previously been described in canine tumours, including osteosarcomas, lymphomas, and mammary tumours (intron motifs of the *CDH2*, *ADAM19*, *CDH5*, and *LRIG1* genes) - Bryan *et al.* [2009], Brandão *et al.* [2018], Noguchi *et al.* [2015], Morimoto *et al.* [2017], Bronzini *et al.* [2017], Nam *et al.* [2020] and Montaner-Angoiti *et al.* [2023]. The lack of differences identified in this study may be attributed to the low variability of the examined regions, as well as the absence of changes in the *tRNA-Leu* (*UUR*) structure, which did not affect the stability of the gene.

In conclusion, the results obtained in this study suggest that the detected polymorphisms in the *tRNA-Leu (UUR)* gene may be characteristic of the Polish dog population. Moreover, the observed alterations may be involved in the process of carcinogenesis in conjunction with accompanying mutations located throughout the genome. The lack of differences in the methylation profiles of the examined tissues may be related to the absence of changes in the structure and stability of the *tRNA-Leu (UUR*) molecule. However, the authors recommend conducting studies on a larger group of individuals.

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