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EVALUATION OF THE *TRNA-LEU (UUR)* GENE HAPLOTYPE PROFILE OBSERVED IN CANINE MAMMARY GLAND TUMOURS BASED ON COMPARATIVE ANALYSIS WITH THE *MT-TL1* HUMAN GENE*

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Abstract

The aetiology and pathogenesis of many canine tumours are likely to be similar to cancers found in humans. This study aimed to evaluate a plausible link between changes in the *tRNA-Leu* (*UUR*) gene and the carcinogenesis process in dogs with mammary gland tumours. The whole mitochondrial DNA (mtDNA) isolated from blood and tumour tissues of 13 dogs with malignant mammary gland tumours was sequenced. The present work is the first report showing that some polymorphisms might occur at the corresponding positions in the human and canine mtDNA genome, which in turn may provoke similar deleterious effects. The homology between the human *MT-TL1* and canine *tRNA-Leu* (*UUR*) genes was 84%. After resequencing of the whole mitochondrial DNA genome with the use of the NGS technology, two polymorphisms in two haplotypes were identified: m.2683G>A (observed in 18 out of 27 samples) and m.2678_2679insG (27 out of 27 samples). The m.2683G>A polymorphism corresponded to a deleterious change at m.3243A>G, which is linked with MELAS (Mitochondrial Encephalomyopathy, Lactic Acidosis, Stroke-like episodes) syndrome and with different types of cancers in humans as well. The comparative analysis of *MT-TL1* and *tRNA-Leu (UUR)* led us to hypothesise that the m.2678_2679insG and m.2683G>A polymorphisms might influence the dog's condition and might be linked with tumourigenesis, as observed in humans.

Key words: polymorphisms, mtDNA, mammary gland tumours, MELAS

Canine malignancies have been established as strong comparative models for human cancers due to their spontaneous development and frequency (Stacey et al., 2007; Grzybowska-Szatkowska and Slaska, 2012; Dobson, 2013). The human mitochondrial DNA genome (mtDNA) is a 16,569 base pair (bp) covalently closed circular molecule containing 13 genes for polypeptides, 2 genes for rRNAs, and 22 genes for tRNAs, whereas canine mtDNA contains the same genes but is 16,727 bp long (Wallace, 2014; Young and Copeland, 2016; Wheeler et al., 2019). The difference between the canine and human mtDNA genomes is the 30 tandem repeats of the 10-nt motif between conserved sequence blocks (CSB) I and II localised in the canine displacement loop (D-loop) region (Tkaczyk et al., 2020). Although the canine mitochondrial genome sequence was described at the end of the 20th century, the association of mtDNA mutations with neoplastic transformation in dogs has not been investigated profoundly to date (Kowal et al., 2019). As in studies of human cancers, the association of mitochondrial DNA mutations with cancer development included both coding and non-coding genes of mtDNA regions (Grzybowska-Szatkowska and Slaska, 2012).

Although functional mitochondria are necessary for cancer cell growth and tumourigenesis, numerous mtDNA mutations and reduced mtDNA copy numbers altering OXPHOS (oxidative phosphorylation) have been reported to be common in cancers (Larman et al., 2012; Wallace, 2012; Singh et al., 2017 a, b). Different modifications of mitochondrial functions have been observed in human and canine cancer biology, including shifts in energy production, disruption of apoptosis signalling, an increase in mtDNA mutations, and altered antioxidant activity and reactive oxygen species production (Ślaska et al., 2013; Singh et al., 2017 c; Surdyka and Slaska, 2017). There are several regions of mitochondrial DNA that have preserved its conservative character in many organisms, e.g. *CYB*, *COI*, and *12s rRNA*, and are widely used as barcodes for identification purposes (Hebert et al., 2010; Kowal et al., 2020). The information about functional regions in human mtDNA is stored in the Mitomap database (Lott et al., 2013); however, as far as we know,

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there is no similar database of canine mtDNA, except for the Mamit-tRNA database for comparison of tRNA genes (Helm et al., 2000). The specific information about the exact localisation of similar functional regions in canine mtDNA is limited and regards mainly the structure and changes in the D-loop (Pereira et al., 2004) for forensic purposes. This paper reports the polymorphisms in two haplotypes in the canine mtDNA *tRNA-Leu* (*UUR*) gene detected in a large-scale analysis of the mtDNA genome from dogs with mammary gland tumours.

The aim of this study was to present a plausible link between changes in two *tRNA-Leu* (*UUR*) gene haplotypes and the carcinogenesis process in dogs with mammary gland tumours, taking into account the comparative analysis of human *MT-TL1* and canine *tRNA-Leu* (*UUR*) in order to find similarities.

Material and methods

We analysed 27 entire mitochondrial genomes. DNA was isolated from thirteen blood samples (n=13) and fourteen post-operative tumour tissues (n=14) obtained from thirteen dogs (one dog had two mammary gland tumours) (Table 1). The dogs obtained neither chemotherapy nor radiotherapy. The tumour tissue sample was placed in a sterile container, and blood was sampled into sterile test-glasses with an anticoagulant. Histopathology analysis of the tumour tissue samples was performed. The malignancy degree of the mammary gland tumour 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). The DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) was used to extract DNA from tumour tissue and blood. DNA samples were assessed quantitatively and qualitatively by spectrophotometric measurement (DeNovix NanoDrop™ Alternative, DS-11) and electrophoretic separation in agarose gel. Selective amplification of mitochondrial DNA was held on the total genomic DNA using two pairs of primers obtained from the literature: F1418 and R11041, ~9,5kb PCR product, 9190F and R2382, ~9,8kb PCR product (Imes et al., 2012). The library preparation and NGS sequencing on an Illumina MiSeq sequencer was performed according to the methodology proposed by Kowal et al. (2019). Full coverage of mitochondrial DNA was obtained after amplification of two long-range PCR products. PCR reactions were carried out using KAPA HiFi PCR Kit reagents (KAPA Biosystems, Wilmington, USA). Mitochondrial DNA was sequenced in the DNA Sequencing and Synthesis Facility (oligo.pl) at the Institute of Biochemistry and Biophysics PAS. 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, CA) following manufacturer's instructions. The library sample was sequenced on an Illumina MiSeq sequencer (Illumina, San Diego, CA) using a 600-cycle kit (v3) in a paired-end mode targeting at least 100x coverage. The data obtained after NGS sequencing was stored in NCBI BioProject number PRJNA679417. The sequence reads were filtered by quality and the remaining adaptors were removed using the fastp tool (Chen et al., 2018). Although the whole mitochondrial DNA sequences were obtained, we focused only on an analysis of the *tRNA-Leu* (UUR) gene in this article. The sequences of mtDNA from the blood and tumour were analysed with the use of bioinformatics programs (Unipro UGENE (v.34.0) (Okonechnikov et al., 2012). The differences in the nucleotide sequence were determined by comparison of the analysed sequences with the reference sequence (GenBank accession No. U96639) (Kim et al., 1998). The comparative analysis of the human and canine *tRNA-Leu* (*UUR*) genes was carried out in the UGENE program by building an alignment of human and dog reference sequence fragments (human Revised Cambridge Reference Sequence GenBank – rCRS – accession No. NC_012920.1). The mtDNA *tRNA-Leu* (*UUR*) genes obtained after sequencing were aligned with CLUSTAL O and MAFFT algorithms in order to obtain the highest similarity between the sequences. The secondary structure of the *MT-TL1* gene was determined using the tRNA-scan program (Lowe and Chan, 2016). The HGVS (Human Genome Variation Society) (2016) nomenclature was used for the description of variants of sequences found in the mtDNA (den Dunnen et al., 2016).

Table 1. Data on dogs with sequenced mitochondrial genomes

Laboratory dog number	Breed/Crossbreed	Sample number		Tumour	Age (years)
1	2	3	4	5	6
S2	Italian Sighthound	S2K S2G	blood tumour	CC	9
S11	Crossbreed	S11K S11G	blood tumour	CC	10
S17	Golden Retriever	S17K S17G	blood tumour	CC	10
S21	Dachshund	S21K S21G	blood tumour	CC	10

Table 1 – contd.

1	2	3	4	5	6
S29	Medium Schnauzer	S29K S29G	blood tumour	CC	11
B131	Polish Lowland Sheepdog	B131K B131G1 B131G2	blood tumour 1 tumour 2	CC	13
S6	Crossbreed	S6K S6G	blood tumour	TPC	9
S7	Prague Ratter	S7K S7G	blood tumour	TPC	14
S19	Siberian Husky	S19K S19G	blood tumour	TPC	9
S23	German Shepherd	S23K S23G	blood tumour	TPC	14
S26	Yorkshire Terrier	S26K S26G	blood tumour	TPC	5
S35	German Shepherd	S35K S35G	blood tumour	TPC	7
S27	Polish Lowland Sheepdog	S27K S27G	blood tumour	CPK	13

CC – carcinoma complexus, TPC – tubulo-papillary carcinoma, CPK – carcinoma planoepitheliale keratodes.

Results

The overall comparative analysis of the human *MT-TL1* gene and the canine *tRNA-Leu (UUR*) gene revealed that the homology rate ranged between 84 and 87%. Dogs with both polymorphisms: m.2678_2679insG and m.2683G>A (haplotype 1) had a higher homology rate compared with the rCRS (87%), whereas dogs with only one polymorphism: m.2678_2679insG (haplotype 2) had a homology rate of 85%. In case of the canine reference sequence from the GenBank database, the homology rate was 84%. Among the two reference sequences, the differences were observed in the following positions (numbering in accordance with rCRS sequence): m.3234, m.3236, m.3239, m.3243, m.3253, m.3254, m.3257, m.3269, m.3276, m.3277, m.3297, and m.3299. The m.2678_2679insG polymorphism was observed in 27 out of the 27 sequences, whereas m.2683G>A was detected in 18 out of the 27 sequences (Figure 1). Haplotype 1 was observed in nine out of the 13 dogs (69%), whereas haplotype 2 was observed in four out of the 13 dogs (31%). None of the analysed samples from the dogs with malignant mammary tumours had the same haplotype as the reference sequence from the GenBank database. All changes were homoplasmic. Haplotype 1 was present equally in dogs with all examined types of mammary gland tumours. We found no mutations in either the blood or the tumour tissue samples.

It is worth noting that the m.2683G>A polymorphism corresponded to the human position m.3243. The mutation at the m.3243A>G position is recognised as a deleterious alteration observed in 80% of patients with MELAS syndrome. We also analysed the secondary structure of molecule of canine (reference sequence) and human (reference sequence) tRNA-Leu (UUR), a representative molecule of tRNA-Leu (UUR) in dogs with haplotype 1, and a representative molecule of human tRNA Leu with the m.3423A>G alteration (Figure 2). The observed alterations did not influence the secondary structure of tRNA, as they were localised between the D-arm and the acceptor stem (m.2678_2679insG) and in the DHU loop (m.2683G>A). The m.3423A>G and m.2683G>A alterations were situated at the same position in the DHU loop. However, the polymorphic transition had opposite directions in the dogs. It is worth noticing that the localisation of guanine at this position in this region in the human molecule is considered normal. However, it should be noted that the structure of *tRNA-Leu* (*UUR*) in the dogs with tumour differed from the reference molecules. Interestingly, the human *tRNA-Leu* (*UUR*) molecule appeared to be more unstable due to the TC>CT transition at positions m.3253 and m.3254 (2693 and 2694 positions in the dog genome) (Figure 2 – green circles).

Interestingly, the *MT-TER* sequence is localised in the same region as the *MT-TL1* gene. *MT-TER* is a 28bp-long transcription terminator area spanning the end of *RNR2* (16s rRNA-coding gene) and *MT-TL1* in human mtDNA (Christianson et al., 1988).

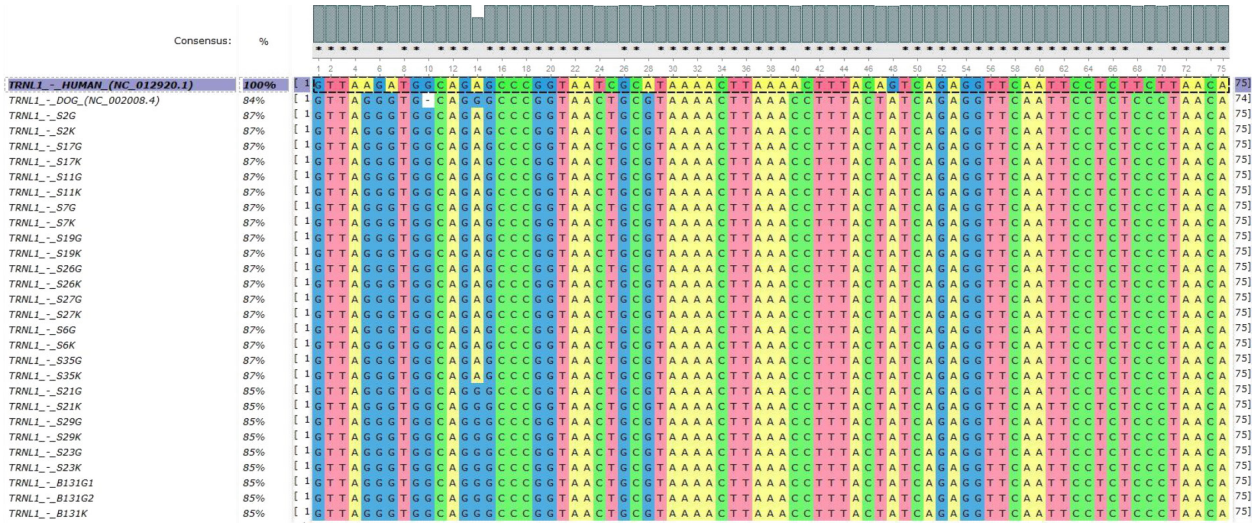


Figure 1. Comparative analysis of *tRNA-Leu (UUR)*and *MT-TL1* genes performed in the Unipro uGene program (Okonechnikov et al., 2012). The reference and examined sequences were aligned with the use of the Clustal O algorithm

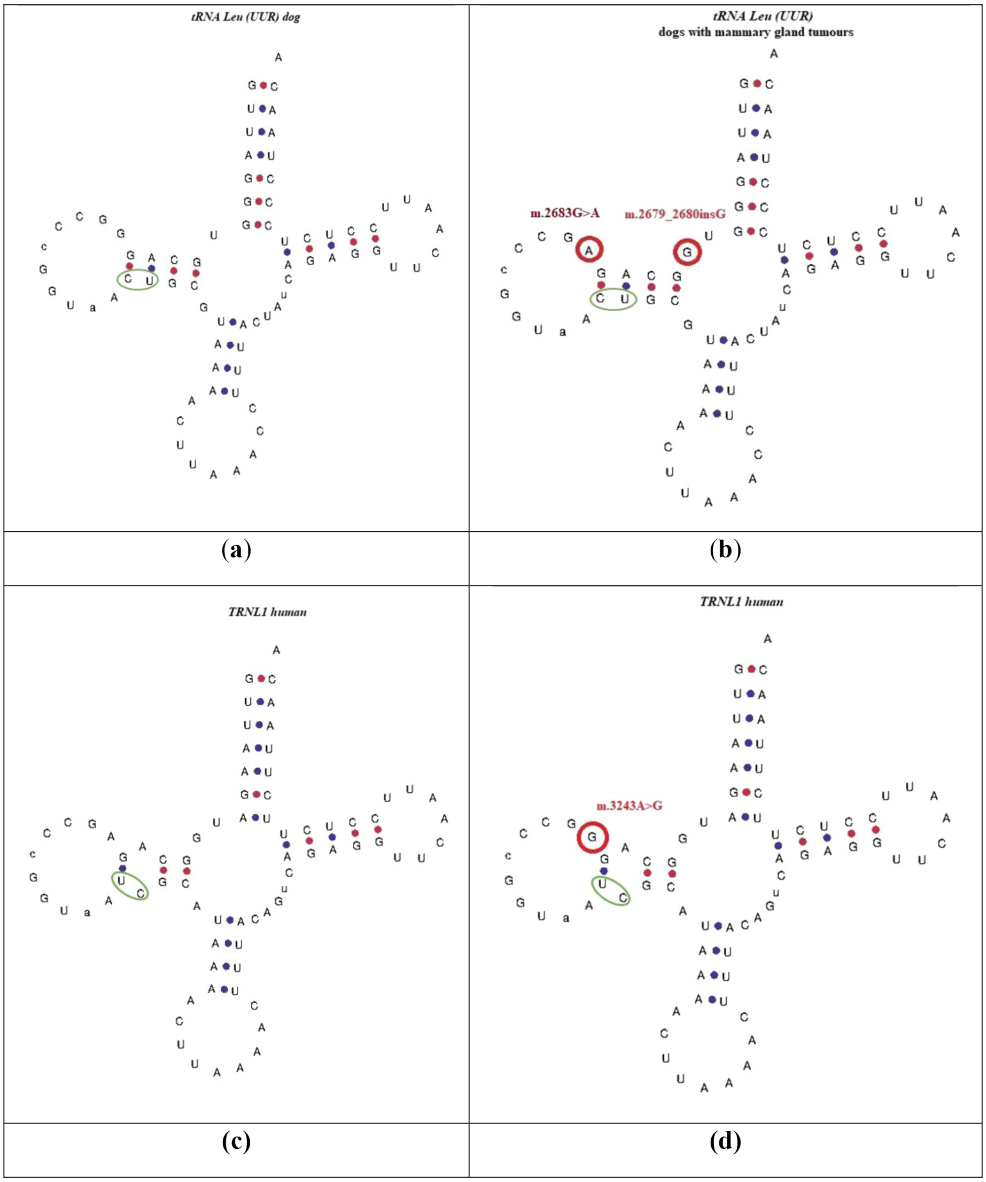


Figure 2. Structures of canine *tRNA-Leu* (*UUR*) human *MT-TL1* obtained from tRNA-scan: (a) Predicted structure of canine *tRNA-Leu* (*UUR*) (reference sequence); (b) Predicted structure of canine *tRNA-Leu* (*UUR*) (analysed mtDNA sequence in dogs with haplotype 1); (c) Predicted structure of human *MT-TL1* (reference sequence) (d) Predicted structure of human *MT-TL1* gene with the m.3243A>G alteration

Discussion

The dog has become a promising model for studying human genetic diseases, including cancers, hence the increased interest in canine genomics (Stacey et al., 2007; Slaska et al., 2014 a). In comparison to humans, dogs are affected by specific types of cancers more frequently: 35 times more often by skin cancer, four times more often by mammary gland cancer, eight times more often by bone cancer, and twice more often by leukaemia (Cullen et al., 2008). Mammary tumours are the second most common canine cancers with a high incidence in female dogs, accounting for 52% of all diagnosed tumours, and over 80% of them are malignant (Ślaska et al., 2013).

To date, most research of alterations in canine mtDNA has been focused on the hypervariable D-loop. The variability in the mtDNA D-loop sequence in dogs with diagnosed mammary tumours was identified in several reports (Grzybowska-Szatkowska and Ślaska, 2014; Slaska et al., 2014 b; Surdyka and Slaska, 2017; Kowal et al., 2019). The D-loop region is responsible for transcription, replication, and organisation of the mitochondrial genome. The single nucleotide polymorphism (SNP) in this region may alter the functions of the electron transport chain and promote the generation of reactive oxygen species, which in turn damage the DNA structure (Brandon et al., 2006; Slaska et al., 2014 b). Kowal et al. (2019) noted the highest rate of heteroplasmy in the VNTR (variable number tandem repeat) region localised in the canine D-loop. Moreover, in the same study, the occurrence of both polymorphisms – m.2679_2680insG and m.2683A>G – was confirmed in the analysis of changes in the whole mtDNA genome obtained from a 9-year-old Labrador with mammary tumour (tubulo-papillary carcinoma) (Kowal et al., 2019).

The knowledge of changes in the mitochondrial DNA in mammary gland tumours is insufficient, and no comparative analysis between the human and canine mtDNA sequences has been done before. Based on the comparative analysis of the human and canine mitochondrial DNA genome, it can be stated that the observed polymorphisms might affect *tRNA-Leu* (*UUR*) and cause pathological changes in the dog's organism or even lead to carcinogenesis. It is worth noticing that the *tRNA-Leu* (*UUR*) gene is responsible for transporting one of the most frequently used amino acids in the mitochondrial protein structure. Mitochondrially encoded proteins are mostly hydrophobic (Brandon et al., 2006). Therefore, it is not surprising that the tRNA transporting hydrophobic amino acids are the most evolutionarily conserved. The changes in the tRNA-Leu (UUR) structure were found between the D-arm and the acceptor stem and in the DHU loop. Determining whether these variants are pathogenic is critical, but confirmation of the effect of the variants on mitochondrial function may be challenging (Bulduk et al., 2020). Sonney et al. (2017) used available databases of the alignment of benign and pathogenic variants between diverse tRNAs, structural information, and comparative genomics to predict the impact of all possible single-base variants and deletions (Bulduk et al., 2020). The highest pathogenicity is mainly observed in the D-stem and Anticodon loops, but it should be emphasised that the disruptions in the D-loops might also affect the tertiary structure of tRNA.

The tRNA genes are crucial for the proper functioning of the OXPHOS chain and for the whole organism. One of the frequently observed mutations (m.3243A>G) in the *MT-TL1* gene in humans causes mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS syndrome) (Bulduk et al., 2020). MELAS syndrome is a multi-organ disease with broad manifestations, including stroke-like episodes, dementia, epilepsy, lactic acidemia, myopathy, recurrent headaches, hearing impairment, diabetes, and short stature (El-Hattab et al., 2015). The most common mutation associated with MELAS syndrome is the m.3243A>G mutation in the *MT-TL1* gene encoding the mitochondrial *tRNA-Leu* (*UUR*) gene. Over 80% of MELAS patients carry the m.3243A>G mutation in the mitochondrial genome (Rahman et al., 2009). The m.3243A>G mutation results in impaired mitochondrial translation and protein synthesis, including the mitochondrial electron transport chain complex subunits, leading to impaired mitochondrial energy production. The inability of dysfunctional mitochondria to generate sufficient energy to meet the needs of various organs results in the multi-organ dysfunction observed in MELAS syndrome (El-Hattab et al., 2015). Even though these symptoms are typical for patients diagnosed with MELAS syndrome, it cannot be confirmed that the same effect would be observed in dogs. The dogs taken to this study were diagnosed with malignant mammary gland tumours; moreover, the direction of the polymorphism is reversed (G>A instead of A>G) as in the MELAS case. However, it should be emphasised that lactic acidosis might appear as a significant biomarker provoked by a genetic alteration in the canine *tRNA-Leu* (*UUR*) gene. Reprogramming of biochemical pathways is a hallmark of cancer cells, and the generation of lactic acid from glucose/glutamine represents one of the consequences of such metabolic alterations (Brown and Ganapathy, 2020). Cancer cells export lactic acid out to prevent intracellular acidification, thereby not only increasing lactate levels but also contributing to an acidic pH value in the extracellular milieu. Lactate and protons in the tumour microenvironment are not innocuous bystander metabolites, but have special roles in promoting tumour-cell proliferation and growth (Brown and Ganapathy, 2020). Queen et al. (2017) suggest a plausible role of the m.3253 and m.3254 variants, which may stabilise the DHU stem and suppress the deleterious effect of the m.3243A>G change (Queen et al., 2017). It is yet unclear whether the same compensatory effect is observed in the reversed change in dogs with the m.2863G>A polymorphism. Although the polymorphism was not observed either in the tumour samples or even in the heteroplasmy

state, it cannot be excluded that it provokes tRNA molecule instability.

The region surrounding m.3243 is an etiologic hotspot for mutations in humans (Moraes et al., 1993). Lorenc et al. (2003) analysed mtDNA variations in various cancer samples by comparing them with normal tissue controls and identified the m.3243A>G mutation in a colon cancer sample and m.3244G>A in lung cancer. A similar mutation was observed by Mayr et al. (2008) and Meierhofer et al. (2006) in studies of renal cell oncocytoma (Meierhofer et al., 2006; Mayr et al., 2008). The latest findings presented by Xu et al. (2019) revealed the presence of m.3243A>G in a non-invasive encapsulated follicular variant of papillary thyroid carcinoma. Thus, it cannot be excluded that m.2683A>G plays a significant role in cancer progression or proliferation in canine malignant mammary gland tumours. However, this hypothesis should be supported by further research conducted on a larger cohort.

The functional *MT-TER* region localised in 3229-3256 bp spans the *MT-RNR1* and *MT-TL1* genes in human mtDNA (Christianson et al., 1986, 1988; Kruse et al., 1989; Hyvärinen et al., 2007). The *MT-TER* region was recognised as a plausible termination transcription region in mitochondrial DNA. Transcription and replication of mtDNA have been regarded as interlinked processes. The primer for initiation of DNA replication has been assumed to be a product of transcription by the mitochondrial RNA polymerase. However, there is no consensus concerning the mechanism by which 3' ends are generated for extension by DNA polymerase, variously proposed to be RNA processing by endonuclease MRP or protein-independent termination at one of the conserved sequence blocks of the non-coding region (NCR) (Hyvärinen et al., 2007). Mitochondrial transcription termination factors (MTERFs) regulate mitochondrial gene transcription and metabolism in numerous types of cells (Sun et al., 2019). Termination at the 3' end of rDNA is brought about by a transcription termination factor mTERF (Christianson et al., 1988; Fernández-Silva et al., 2003), which has also been proposed to interact with the RNA polymerase in initiation site selection (Martin et al., 2005). However, the mechanism of termination of heavy strand promoter (HSP) transcription is still unclear. It was previously suggested that mitochondrial termination factor 1 (MTERF1) bends the mtDNA connecting the HSP1 promoter site and its apparent *tRNA-Leu* (*UUR*) termination site. MTERF1 would then induce transcription termination through base flipping and DNA unwinding. This model was originally proposed to explain the 50-fold higher abundance of mitochondrial rRNAs. However, more recent evidence contradicts this hypothesis (D'Souza and Minczuk, 2018). Yet, it should be emphasised that the *MT-TER* region in humans has a high homology rate compared with the corresponding canine mtDNA region (unpublished data – 79% comparing human and canine reference sequences and 86% comparing the human reference sequence and the sequence obtained from dogs with mammary gland tumours with two *tRNA-Leu* (*UUR*) polymorphisms). It is worth underlining that changes in this region might alter mitochondrial DNA transcription and replication in dogs, leading to disruptions that may provoke cancer progression and proliferation.

Conclusions

The comparative analysis of the human and canine mtDNA genome revealed a high homology rate (84–87%) in the *MT-TL1* gene. The presence of the canine polymorphism m.2683G>A corresponded to the human m.3243A>G deleterious alteration, which is frequently diagnosed in MELAS syndrome patients. Moreover, such an alteration in humans has frequently been linked with different types of cancers, i.e. colon cancer, renal cell oncocytoma, and papillary thyroid carcinoma. Thus, it cannot be excluded that the presence of the polymorphisms in canine *tRNA-Leu* (*UUR*) might be linked with mammary gland tumours. It is also worth mentioning that the polymorphisms are located in the plausible *MT-TER* region in dogs responsible for transcription termination. However, the association of these two polymorphisms with the carcinogenesis process cannot be clearly confirmed. The authors emphasise that this research is based on bioinformatics analysis, and a further experiment based on additional canine cancer samples should be carried on for full confirmation of the hypothesis and to evaluate the rate of association of the identified mitochondrial mutation with the occurrence of mammary gland tumours or other cancers in dogs.

Ethics approval

The study was approved by the II Local Ethical Commission for animal experiments in Lublin, Poland (resolution number 6/2013).

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