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Mitochondrial D-loop mutations can be detected in sporadic malignant tumours in dogs

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Abstract

The aim of this study was to identify mutations in the D-loop region of mtDNA in head, neck, and limb tumours in dogs, and determination of their relationship with the process of neoplastic transformation. Blood and tumour tissue samples from 19 dogs with diagnosed sporadic malignant tumours were analysed. DNA extraction, amplification, and sequencing of the mtDNA D-loop, and bioinformatic analyses were performed. Five mutations and 19 polymorphisms were observed in 68.42% of all tumours. Polymorphic variants were noted in 42.86% of the head and neck tumours and in 58.33% of the limb tumours. Mutations were observed in 21.05% of dogs. The mutations were found in 28.57% of the head and neck tumours and in 16.66% of the limb tumours. The mutations were identified in 50% of the studied epithelial cancers. In the mesenchymal tumours, no mutations in the D-loop region were observed. Mitochondrial haplotype A17 was found in over 40% cases of limb tumours. No association between the age, breed, sex, type of tumour, and detected polymorphic variants were observed. Different mutational changes in the D-loop sequences of mtDNA identified in the blood and tumour tissues may indicate a relationship between the type of tumour and individual changes in the D-loop nucleotide sequences of mtDNA.

Keywords: dog, tumour, D-loop, mtDNA, mutations.

Introduction

According to Kim *et al.* (16), the complete nucleotide sequence of the domestic dog mitochondrial genome consists of 16727 bp, and the non-coding control region (mtCR) or D-loop is located between positions 15458–16727 (1270 bp), where the presence of a heteroplasmic repeat unit divides the mtCR into two subsections, hypervariable region I (HVI) and hypervariable region II (HVII) (15, 23).

Analysis of mitochondrial DNA (mtDNA) sequences plays an important role in the studies on population genetics and in medicine, including veterinary medicine (7, 11, 12, 27-29, 32). Results published in the recent years have indicated the prevalence of mitochondrial DNA mutations in tumour cells, which suggests that they may be cancer-causing agents (7, 11, 12, 28, 29, 32, 34). Up till now, there has been not stated whether the observed changes in mitochondrial DNA result from the development of tumour or are connected with the process of neoplastic transformation (7, 11, 12, 28, 29). Grzybowska-Szatkowska and Slaska (12) report the presence of homological changes in the sequence of mtDNA in breast cancer and some mitochondrial diseases, which indicates that mutations in mtDNA in breast cancer may affect the cell and cause its dysfunction, as in the case of mitochondrial diseases.

The molecular basis of many human tumours attracts much more attention than in the case of animal tumours. Growing expectations of breeders and dog owners with regard to diagnosis and treatment encourage scientists to undertake extensive research in this area. Rowell *et al.* (25) report that about 400 diseases in dogs and humans are analogous in the genetic background and course. Certain types of

tumours, such as canine mammary carcinoma, are genetically, morphologically, clinically, and prognostically related to human tumours. Hence, the dog is a suitable animal model for naturally occurring tumours (18, 24, 25, 31-33).

The studies on tumour tissues showed a significant number of somatic mutations in mtDNA (3, 7, 11, 12, 22, 28, 29), especially in the D-loop region which controls replication and transcription of mitochondrial DNA. These mutations can lead to disorders in these processes and cell energy functions (7, 11, 17). Therefore, the mutations identified in the D-loop region may affect the mitochondrial electron transport chain, which is responsible for release of reactive oxygen species (ROS). High level of ROS may lead to changes in the mitochondrial genome and initiation of tumour growth (7, 11, 32, 34).

The breed specific predispositions to malignant tumours in dogs indicate a significant genetic component of neoplastic transformation. In many cases, the aetiology of human and canine genetic diseases is similar because of the orthology of genes, in which mutations causing genetic disorders in the process of malignant transformation are located (32). Identification of risk factors may increase prognostic and diagnostic possibilities in the case of human and canine tumours. Somatic mutations in mtDNA have been described in many types of human tumour cells (7, 8, 11-13, 17, 20, 21, 34), but there are only a few literature reports documenting the occurrence of mutations in the mtDNA of dogs with tumours (3, 22, 28, 29, 32). Mutations may occur in the entire mitochondrial genome but, most often, they occur in the hypervariable regions of the D-loop (3, 22, 28, 29, 32). Therefore, an attempt is made to identify mutations in the mitochondrial D-loop and connect them with the process of carcinogenesis in dogs.

Dog's clades are marked with the use of capital letters (from A to F) (26). Networks reported by Savolainen *et al.* (26) show sub-hierarquisation especially within clade A, and the further indication of the positions will make it possible to easily classify sub-clades. According to Imes *et al.* (15), 10 most commonly observed mtGenome HVI haplotypes (A2, A11, A16, A17, A18, A19, A22, A26, B1, C3) compose approximately 53% of the sequenced canine mtDNA. The remaining mtGenomes exhibit one of 118 HVI haplotypes. Phylogenetic analysis of Savolainen *et al.* (26) assigned the dog sequences into the same four phylogenetic groups (clades A, B, C, and D) and to clades E and F, consisting of an isolated haplotype. Haplotypes of clades D, E, and F were found only regionally (26). A total of 71.3% of all dog populations worldwide has haplotypes belonging to clade A, and 95.9% has the types belonging to clades A, B, or C. Clade A has been shown to be represented in all geographic regions, and clades B and C in all regions except America. The frequencies of clades A, B, and C are similar in all regions and there is no clear division of the main morphologic types of dogs or of large and small breeds among the three main clades (26).

In the literature there is no information about the relationship between dog's mitochondrial haplotypes and neoplasia in dogs. The most common and most widely studied tumours in dogs are breast cancers as well as head and neck tumours. Other types of tumours, such as gastrointestinal and limb tumours occur less frequently (4, 10, 19, 30, 32, 35). There are no reports about genetic determination of limb tumour. The aim of this study was the identification of mutations in the D-loop region of mtDNA in head, neck, and limb tumours in dogs and determination of their relationship with the process of neoplastic transformation.

Material and Methods

Thirty-eight samples obtained from blood (n = 19) and tumour tissues (n = 19) of 19 dogs of 11 different breeds and six crossbreeds were analysed. The breed specifications and malignant tumour types of preliminary studies are given in Table 1.

The dogs received no chemotherapy or hormone therapy. Analyses were performed on DNA isolated from post-operative tissues. Tumour tissues were collected along with blood.

Prior to DNA isolation, the histological type of the individual tissue was routinely assessed. Sections were sampled from each surgically removed tumour for histopathological analyses. The tissues were fixed in buffered formalin, embedded in paraffin blocks, and preparations were stained with haematoxylin and eosin. Microscopic assessment of tumours was performed in accordance with the WHO histological recommendations (International Classification of Tumours of Domestic Animals).

DNA was extracted from tumour tissues and their matched blood samples using the DNeasy Blood & Tissue Kit (Qiagen). DNA samples were assessed quantitatively and qualitatively by electrophoretic separation in agarose gel and spectrophotometrically by measurements of sample absorbance in a BioPhotometer (Eppendorf, Germany) spectro-photometer.

Amplification of the non-coding mitochondrial DNA region, *i.e.* the D-loop, was performed using the PCR technique in a MJ Research PTC 225 Tetrad thermal cycler. Primers used in the analysis, encompassing a mtDNA fragment between nucleotide 15341 and 15804 (LF 5`- TTACCTTGGT CTTGTAAACC; HR 5`- CTGAAGTAAGAACCA GATGCC), were found in literature reports (2). The annealing temperature (Ta - 48ºC) and amplification conditions were established experimentally. Amplification products were visualised on 2% agarose gel. Amplicons were sequenced using a BigDye Terminator Cycle Sequencing kit (Applied Biosystem, USA) in GeneAmp PCR system 9700 (Applied

Biosystem, USA). The samples were subsequently purified on CentriSep columns according to the manufacturer's protocol or precipitated with ethanol and sodium acetate according to the protocol of the BigDye kit manufacturer. Extension products were separated on an ABI 377 automated sequencer (Applied Biosystem, USA).

The nucleotide sequences obtained were subjected to bioinformatic analyses (1, 14) in order to determine mutation and polymorphic sites within the analysed mtDNA fragment in each of the tissues sampled from the individuals and in the groups of head, neck, and limb tumours (9). The D-loop nucleotide sequence in all analysed groups was compared to the reference sequence (GenBank accession No. U96639; 16). HVI dog haplotype have been established according to Savolainen *et al*. (26), Imes *et al.* (15), and Pereira *et al.* (23). The polymorphisms involved changes which occur in blood and tumour cells in the same dog. Mutation is a change characteristic only for tumour cells and not occurring in dog's blood.

The study was approved by the II Local Ethical Commission for Animal Experiments in Lublin, Poland.

Results

Among the studied tumours, mastocytoma constituted the largest group (21%). Haemangiopericytoma was the most common neoplasia (25%) in the group of limb tumours (Table 1). Tables 2-4 show the results of analysis of D-loop sequences in different tissues of dogs with diagnosed tumours.

Table 1. Characteristics of the studied tumour types in dogs

Laboratory sample number	HVI dog haplotype	Origin of the tumour	Tumour type	Location	Breed/ crossbreed	Sex	Age (years)
Head and neck tumours							
26	A1	Epithelial	Carcinoma glandulae thyroideae	Thyroid	Crossbreed	M	15
27	A18	Epithelial	Adenoma glandulae sebacei	Ear	German Shepherd	M	2
33	B1	Epithelial	Carcinoma sebaceum	Ear	Spaniel	M	14
37	A18	Mesenchymal	Mastocytoma G2	Upper lip	Central Asian Shepherd Dog	F	4
51	A11	Epithelial	Epithelioma sebaceum	Eyelid	York	F	5
64	A18	Mesenchymal	Osteosarcoma	Jowl	Rottweiler	M	11
81	A17	Mesenchymal	Mastocytoma G2	Neck	Labrador	M	10
Limb tumours							
28	A22	Epithelial	Carcinoma planoepithelialae keratodes	Finger	Crossbreed	M	8
31	A17	Mesenchymal	Mastocytoma G1/G2	Buttock	Golden Retriever	F	4
44	B1	Mesenchymal	Lipoma	Buttock	Schnauzer	F	10
47	A167	Mesenchymal	Haemangiopericytoma	Ankle	Crossbreed	M	9
52	A18	Mesenchymal	Mastocytoma G2	Metatarsus	Crossbreed	F	8
55	A18	Epithelial	Carcinoma hidradenoides	Finger	German Shepherd	M	5
58	A18	Epithelial	Carcinoma basocelullare	Wrist joint	German Shepherd	M	8
75	A17	Mesenchymal	Haemangiopericytoma	Elbow	Crossbreed	M	7
80	A17	Mesenchymal	Haemangiopericytoma	Buttock	Crossbreed	F	16
85	A17	Epithelial	Carcinoma planoepitheliale	Finger	Mastiff	M	5
91	A17	Mesenchymal	Haemangiosarcoma	Knee	Pointing breed	M	12
95	A18	Mesenchymal	Histiocytic sarcoma	Metatarsus	Polish Tatra Sheepdog	M	13

Table 2. Differences in the mitochondrial D-loop region between the reference sequence and blood and tumour tissue sequences in the group of head and neck tumours

Sample number	Tumour type	HVI haplotype	Reference sequence	Sequences in dogs' blood	Sequences in dogs' tumour cells
26	Carcinoma glandulae thyroideae	A1	15627T 15639A	15627C 15639T	15627C 15639T
33	Carcinoma sebaceum	B1	15435C 15526G 15595G 15612A 15632G 15639A 15643T 15652C	15435T 15526A 15595A 15612G 15632A 15639C 15643C 15652T	15435T 15526A 15595A 15612G 15632A 15639C 15643C 15652T
81	Mastocytoma G2	A17	15620A 15627T 15639A	15620G 15627C 15639T	15620G 15627C 15639T

Table 3. Differences in the mitochondrial D-loop region between the reference sequence and blood and tumour tissue sequences in the group of limb tumours

Sample number	Tumour type	HVI haplotype	Reference sequence	Sequences in dog's blood	Sequences in dogs' tumour cells
28	Carcinoma planoepithelialae keratodes	A22	15475A 15627T 15632G 15639A	15475G 15627C 15632A 15639T	15475G 15627C 15632A 15639T
31	Carcinoma sebaceum	A17	15620A 15627T 15639A	15620G 15627C 15639T	15620G 15627C 15639T
44	Lipoma	B1	15435C 15526G 15553T 15595G 15612A 15632G 15639A 15643T 15652C	15435T 15526A 15553C 15595A 15612G 15632A 15639C 15643C 15652T	15435T 15526A 15553C 15595A 15612G 15632A 15639C 15643C 15652T
47	Haemangiopericytoma	A167	15372C 15435C 15508G 15526G 15611A 15639A 15650A 15710G	15372T 15435T 15508A 15526A 15611G 15639C 15650G 15710A	15372T 15435T 15508A 15526A 15611G 15639C 15650G 15710A
75	Haemangiopericytoma	A18	15620A 15627T 15639A 15665A	15620G 15627C 15639T 15665G	15620G 15627C 15639T 15665G
80	Haemangiopericytoma	A17	15620A 15627T 15639A	15620G 15627C 15639T	15620G 15627C 15639T
85	Carcinoma planoepitheliale	A17	15620A 15627T 15639A	15620G 15627C 15639T	15620G 15627C 15639T

Table 4. Differences between blood and tumour tissue in the D-loop fragment in head, neck, and limb tumours

Sample number	Tumour type	Sequences in dogs' blood	Sequences in dogs' tumour cells	HVI haplotype
26	Carcinoma glandulae thyroideae	15483A	15483G	A1→A17
		15620A	15620G	
51	Epithelioma sebaceum	15639T	15639A	A11→A18
55	Carcinoma hidradenoides	15529G	15529G/A	A18→A181*
58	Carcinoma basocelullare	15627T	15627C	A18→A17

* Newly described haplotype.

Analysis of sequences of the mtDNA hypervariable region in dogs with tumours showed a relatively high level of genetic variability. Mutations and/or polymorphisms in the D-loop fragment were found in 68.42% cases of tumours. Substitutions relative to the reference sequence were identified in 52.6% of all analysed tumours (Tables 2, 3). Polymorphic variants were noted in 42.86% of the head and neck tumours and in 58.33% of the limb tumours. Mutations were observed in 21.05% dogs with tumours (Figs 1, 2). These mutations were found in 28.57% of the head and neck tumours and in 16.66% of the tumours of limbs. All tumours with mutations were epithelial neoplasms, and the mutations occurred in 50% of epithelial cancers (Tables 1, 4). In the studied material, majority of the detected changes were homoplasmic. Heteroplasmy was observed in one case of limb epithelial cancer – carcinoma hidradenoides (Table 4, Fig. 2). Mitochondrial haplotype A17 was found in over 40% cases of limb tumours (Table 1).

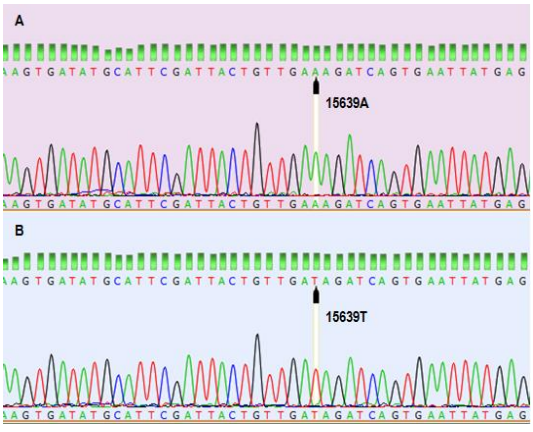


Fig. 1. Blood and tumour mutation in epithelioma sebaceum (A - tumour tissue; B - blood)

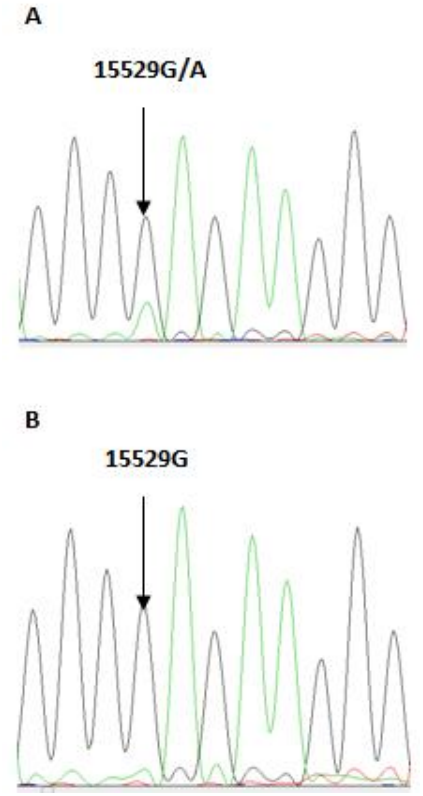


Fig. 2. Blood and tumour heteroplasmy in carcinoma hidradenoides (A - tumour tissue; B - blood)

A change in the position 15 639 was identified in 57.90% of all tumours. Polymorphism A15627G was also frequently observed (36.84% of tumours). The changes occurred in different tumours, independently of the occurrence site and origin (Tables 2, 3). Substitutions in positions T15475C and T15665C were identified in carcinoma planoepithelialae keratodes and haemangiopericytoma (Table 3). In eight positions (G15435A, C15526T, C15595T, T15612C, C15632T, T15639G, A15643G, G15652A), the changes occurred in carcinoma sebaceum and lipoma. Polymorphisms, which accounted for 61.1% of all the polymorphic changes observed, were identified in all dogs with diagnosed haemangiopericytoma tumour.

In the group of head and neck tumours, polymorphism T15639A was observed in carcinoma glandulae thyroideae and mastocytoma G2 (Table 2) and polymorphism T15639G was detected in carcinoma sebaceum. Majority of polymoprhisms were identified in the case of carcinoma sebaceum (Table 2). Mutations were noted in carcinoma glandulae thyroideae (A15483G, A15620G) and epithelioma sebaceum (T15639A) (Table 4, Fig. 1). These sequences differed in the tumours and blood. In the case of three tumours of the head and neck (27, 37, 64), there were no differences between the D-loop sequence and the reference sequence (Tables 1, 2, 4).

In group of limb tumours and in group of head and neck tumours, the change in position 15 639 was observed in all individuals with polymorphisms (Table 3). In all angioma tumours, which occurred only in limbs, many changes were identified in the D-loop region. The mutations were noted in carcinoma hidradenoides (G15529G/A) and carcinoma basocelullare (T15627C) (Table 4). Heteroplasmy was identified in tumour tissue (G15529G/A). In this case, correct (G15529) nucleotide sequence was noted in blood (Fig. 2).

In the case of three tumours of limbs (52, 91, 95), there were no differences between the D-loop sequence and the reference sequence (Tables 1, 3, 4).

Discussion

Defects in mitochondrial DNA have been studied for many years in order to determine their role in progression of cancer. The differences in energy metabolism of tumour and normal cells are the biochemical basis for speculation about the therapeutic strategies, which in the future can be used for selective killing of tumour cells (5). Previous studies concerned mainly mutations in human mtDNA in carcinogenesis (7, 11, 12, 34). Only a few authors have conducted investigations on the relationship between mutations and carcinogenesis process in dogs (3, 22, 28, 29).

Bertagnolli *et al.* (3) analysed mixed breast tumour in dogs and revealed a few cases of substitutions in the D-loop region of mtDNA. Majority of them were heteroplasmy. The sequence of the D-loop was also used as one of the molecular markers to identify the clonal origin and evolution of canine transmissible venereal tumour from different continents (22). Our previous studies on polymorphic changes in the D-loop region in dogs with tumours identified seven polymorphic changes in dogs with malignant tumours (29). Heteroplasmy was also observed. The analyses demonstrated polymorphic changes in nearly 45% of the studied individuals. Continued research in the group of dogs with head and neck tumours and limbs tumours also showed a large number of mutations and polymorphic changes (Tables 2-4). Five changes in positions 15 483, 15 620, 15 627, 15 639, and 15 643 were identified both in our present (Tables 2-4) and previous studies (29).

The presence of more than one type of mtDNA in an organism (heteroplasmy) is the result of recombination in mtDNA (8). Heteroplasmy was found in this study in carcinoma hidradenoides (Table 4, Fig. 2). Investigations on changes in the nucleotide sequence of the D-loop in oral tumours were conducted by Lin *et al.* (20). Mutations were identified in 64% of patients; 68.4% of these mutations were heteroplasmic. There was no relationship between the studied factors (age, sex), tumour localisation, histopathological features, stage, smoking, alcohol consumption), and groups. In contrast to that, the present study indicated the occurrence of only one heteroplasmy in the

nucleotide sequence in position G15529G/A (Table 3). The presence of this change in only one individual may be related to the very low frequency of heteroplasmy in tumour cells in dogs.

All mutations and polymorphic changes observed in the present study were single nucleotide substitutions (Tables 2-4). A study on six cell lines of squamous head and neck tumour was conducted by Challen *et al.* (6). Analysis showed the presence of six substitutions in the HVS1. The authors found no association between the identified changes and the age, sex, tumour localisation, and stage of tumour. In this paper, no relationship between the age, breed, sex, tumour type, and the localisation of polymorphic changes in the nucleotide sequence of the D-loop was observed. Similar results were shown by Lin *et al.* (20) and Challen *et al.* (6). All tumours with mutations were epithelial cancers (Tables 1, 4). Mitochondrial haplotype A17 was found in over 40% cases of limb tumours (Table 1). Moreover, no relationship between the haplotype and the tumour type was observed (Tables 1-4).

Studies on mutations in the D-loop sequence were also conducted by Lièvre *et al.* (21). Their analyses involved patients with head and neck tumours. Twenty-five mutations were identified in 23 individuals. These mutations were mostly several basis-pair deletions and insertions. In our present and previous research (28, 29), no deletions or insertions were identified in malignant tumours in dogs.

This study demonstrated the occurrence of 19 polymorphic changes in 53% of tumours. The most common polymorphism was substitution in position 15639 (substitution T/A or T/G). The association between changes in the sequence of the D-loop region of mtDNA and larynx tumour was identified by Guo *et al.* (13). In total, 34 mutations in the hypervariable region of the D-loop were identified in more than 50% of the studied individuals. In addition, 86 polymorphisms were identified. In the present study, five mutations and many polymorphic changes were observed (Table 4). All tumours with mutations were epithelial cancers, irrespective of tumour localisation. These mutations occurred in 50% of all studied epithelial cancers.

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