Identification of additional mitochondrial DNA mutations in canine mast cell tumours

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Abstract

Background: Research has revealed the presence of somatic mutations in mitochondrial DNA (mtDNA) of certain types of tumours. As this has not been studied for canine mast cell tumours, the aim of this study was to identify mutations in the hypervariable region of mtDNA in mast cell tumours in dogs and determine their association with the process of neoplastic transformation.

Results: Samples from 17 dogs with histopathologically confirmed mast cell tumours were analysed. The samples consisted of tumour tissues (n = 17), normal tissues (n = 17), and blood (n = 17). Amplicons of the displacement loop (D-loop) were sequenced and the obtained nucleotide sequences were subjected to bioinformatics analyses. Somatic mutations were detected in seven positions of the D-loop nucleotide sequences in 47% of the dogs, while polymorphisms were identified in 94% of the dogs. Most of these changes were homoplasmic, while heteroplasmy was detected in two individuals. Six new haplotypes were established as being characteristic for canine mast cell tumours. There was no association between the presence of the mutations and sex, haplotype, or malignancy grade assessed in 3 and 2-grade scales.

Conclusions: Differences in the frequency of somatic mutations imply their direct association with the neoplastic transformation. However, their functional consequences and clinical significance are not clear. The mutations may be used for diagnosis and prognosis of canine mast cell tumours in the future.

Keywords: Tumour, Dog, D-loop, mtDNA, Mutations

Findings

Mast cell tumours account for 7–21% of all diagnosed skin neoplasms in dogs [1]. The aetiology of mast cell tumour development is not fully understood but recent research of human tumours has revealed the presence of somatic mutations in mitochondrial DNA (mtDNA), in particular in the D-loop region, which controls replication and transcription of mtDNA [2, 3].

Somatic cells contain hundreds to several thousand mitochondria, each containing 1–10 gene copies of mtDNA, which often undergoes spontaneous mutations due to their lack of protective activity of histones, the globular, coiled structure of mtDNA and a high level of mitochondrial reactive oxygen species (ROS). When a cell or tissue contains both mutated and normal wild-type mtDNA, the condition is known as heteroplasmy, while homoplasm refers to the presence of only one type of mtDNA (mutated or normal mtDNA). Most changes within the mtDNA nucleotide sequences of neoplastic cells are homoplastic [3].

Investigations of the association between mtDNA somatic mutations and neoplastic transformation have primarily been conducted in humans [2–4] where somatic mtDNA mutations have been found in malignant tumours such as prostate and breast cancer. Such mutations are considered to trigger the neoplastic...
DNA samples were assessed quantitatively and qualitatively by electrophoretic separation in agarose gel, spectrophotometrically by measurements of sample absorbance in a BioPhotometer spectrophotometer (Eppendorf, Hamburg, Germany). Amplification of the D-loop was performed, using a polymerase chain reaction (PCR) technique in a T100 Thermal Cycler (Bio-Rad, Wroclaw, Poland). Primer sequences used in the analysis, encompassing a mtDNA fragment between nucleotide position 15746–16107 (LF 5′- CATACTAACGTGGGTTTAC; HR 5′- CCATTGACTGAAATAGCACCTTG), were based on already published data [13]. The annealing temperature (Ta-60.8 °C) and amplification conditions were established experimentally. Amplification products were visualized on 2 % agarose gel. Amplicons were sequenced using a BigDye Terminator Cycle Sequencing kit (Applied Biosystem, Foster City, CA, USA) in GeneAmp PCR system 9700 (Applied Biosystem). 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).

The obtained nucleotide sequences were subjected to bioinformatics analyses in order to determine mutations and polymorphic sites within the mtDNA fragment of each sample (DNA Baser Sequence Assembler v 3.2). The D-loop nucleotide sequences were compared to the reference sequence [GenBank: U96639] [14, 15] and described as the polymorphisms. The hypervariable region I (HVI) dog haplotypes were established according to Savolainen et al. [16], Pereira et al. [17], and Imes et al. [18].

The probability of the presence of a mutation in each locus in relation to the age, sex, haplotype, and malignancy grade was estimated using the method of least-squares means (lsmeans) ± standard error (se). The correlation between the data was analysed using the SAS 9.4 procedure PROC GLM (SAS Institute, Cary, NC, USA). Correlations with P ≤ 0.05 were considered significant.

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

Data on sex, breed, age and malignancy grade of the dogs are presented in Table 1. Mutations and/or polymorphisms were observed in eight positions of the D-loop nucleotide sequences in all cases (n = 17) of mast cell tumours (Tables 2, 3). Somatic mutations in seven positions of the D-loop nucleotide sequences were detected in 47 % of the dogs (Table 2). All changes were substitution mutations, i.e. exchange of one nucleotide pair with another. These mutations were detected in the tumour tissue and blood, but not in the normal tissues. The majority of the changes were homoplasmic. In one dog, heteroplasmy was detected in the blood and tumour in position m.C15815C/T (Table 2); in another dog, heteroplasmic changes were found only in the
tumour in positions m.C15912T/C and m.C16025T/C (Fig. 1). Somatic mutations in tumour tissues in positions m.16003 (Fig. 2) and m.15912 mtDNA exhibited the highest frequency, in approximately 35 and 24 % of the cases, respectively. These mutations were also detected in the blood of one dog.

Recently reported tumour haplotypes (except B11) were present in tumour tissue but not in normal tissue or blood (Table 2). The HVI dog haplotype A18, accounting for 41.7 % in normal tissue and blood, was the most prevalent haplotype in each type of the analysed tissues. In tumour tissue, it was present in 41.2 %
of the tumours. The mtDNA haplotype was changed in all dogs with detected mutations; hence, six not previously reported haplotypes of mast cell tumours were detected. Compared to the reference canine sequence, polymorphisms (m.T15800C, m.C15814T, m.T15815C, m.C15912T, m.C15955T, m.A16003G, m.T16025C) were found in seven positions of the D-loop sequences in 16 of 17 dogs (94 %) with mast cell tumours (Table 3). In most dogs (94 %) polymorphism was detected in position m.C15814T, while polymorphism at D-loop position m.C15955T was found in 35 % of the examined dogs (Table 3).

Mutations in loci m.15912 and m.16003 were only detected in dogs aged six year or older (age groups II and III). The risk of mutation in locus m.16003 was significantly higher in the oldest dogs (age group III) compared to the youngest dogs (P = 0.047). However, since the group sizes were limited, these data should be interpreted with care. Statistical analyses did not reveal an association between presence of mutations and sex, haplotype, or the tumour malignancy grades (Tables 1 and 2).

Analysis of the D-loop sequences revealed a relatively high genetic variability. As the mutations occurred independently of the mast cell tumour malignancy grades, this indicates that the presence of mutations in the D-loop and the malignancy grade was not associated. Mutations in the mtDNA sequences and sex or haplotype were not associated as also observed for other types of canine tumours [7–9]. Some of the observed mutations (except m.16003 and m.16025) have been recognized in other types of canine tumours previously [5]. These were mostly homoplasmic substitution mutations although single heteroplasmy mutations were found as well.

| Dog number | Reference sequence | Sequence in normal cells | Sequence in blood | Sequence in tumour cells |
|------------|--------------------|--------------------------|------------------|--------------------------|
| 1          | m.15800T           | m.15800C                 | m.15800C         | m.15800C                 |
| 1–12, 14–17| m.15814C           | m.15814T                 | m.15814T         | m.15814T                 |
| 7          | m.15815T           | m.15815C                 | m.15815C         | m.15815C                 |
| 1, 7       | m.15912C           | m.15912T                 | m.15912T         | m.15912T                 |
| 1, 4–7, 12 | m.15955T           | m.15955T                 | m.15955T         | m.15955T                 |
| 1          | m.16003A           | m.16003G                 | m.16003G         | m.16003G                 |
| 14         | m.16025T           | m.16025C                 | m.16025C         | m.16025C                 |

Differences were not present in one of the studied dogs (no. 13)

Table 3 Differences in the mitochondrial D-loop region between the reference sequence and normal tissue, blood and tumour tissue sequences in cases of canine mast cell tumours

Fig. 1 Cancer heteroplasy in position m.16025 (a normal tissue, b blood, c tumour tissue)
Presence of mutations in the D-loop sequences in tumour tissue and/or blood of dogs with mast cell tumours indicates that they may be involved in the process of neoplastic transformation. Further studies on the significance of mtDNA D-loop mutations and their association with neoplastic transformation, biological behaviour, and histologic grade of canine mast cell tumour are however required to determine their significance.

Authors’ contributions
AS and BS participated in the design of the study, analyses and drafted the manuscript. MS participated in molecular genetic study. LGSz participated in the analyses of the results. WL performed the histopathological examination. DR collected samples and data on breed, age and sex of dogs. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

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