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False-positive insertions detected by Oxford Nanopore Technology in homopolymeric regions of mitochondrial cytochrome coxidase subunit genes: a study of selected canine cancer cases

BARTŁOMIEJ KIECAK, BLANKA JÓŹKÓW, KAJA ZIÓŁKOWSKA-TWAROWSKA, PAWEŁ GRYCHNIK, ANGELIKA TKACZYK-WLIZŁO, BRYGIDA ŚLASKA, KRZYSZTOF KOWAL

Institute of Biological Bases of Animal Production, University of Life Sciences in Lublin, ... Lublin, Poland

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Kiecak B., Jóźków B., Ziółkowska-Twarowska K., Grychnik P., Tkaczyk-Wlizło A., Ślaska B., Kowal K.
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Summary

The aim of this study was to validate insertions located in homopolymeric regions of the *MT-CO1*, *MT-CO2*, and *MT-CO3* genes, which were identified as potential false positives following Oxford Nanopore Technology (ONT) sequencing of canine mitochondrial DNA (mtDNA). The analysis included 22 blood and tumor tissue samples collected from five dogs with primary, recurrent and metastatic tumors. Sanger sequencing was employed to verify these variants. In total, 41 positions in homopolymeric regions were identified, where false insertions were systematically reported. Sanger sequencing did not confirm the presence of any of the detected changes in the examined regions, definitively classifying all observed variants as technical artifacts resulting from the specifics of nanopore sequencing.

Keywords: mtDNA, DNA sequencing, Oxford Nanopore Technology, homopolymeric regions

Current knowledge of the genetic aetiology of cancer is focused primarily on molecular alterations in nuclear DNA (nDNA). However, recent scientific reports have increasingly emphasized the role of mitochondrial DNA (mtDNA) as an important component of oncogenesis (7). In veterinary science, this area is still poorly understood, and comprehensive studies are particularly limited. Although changes in the mitochondrial genomes of dogs with malignant tumors have been identified (10, 20, 21), the role of these alterations has not yet been sufficiently investigated in the context of tumor recurrence and metastasis.

The *MT-CO1*, *MT-CO2* and *MT-CO3* genes are located in mtDNA and encode proteins that form part of cytochrome c oxidase (complex IV of the respiratory chain), an enzyme of the mitochondrial electron transport chain. Their correct expression is essential for the efficient oxidative phosphorylation, in which energy derived from oxygen reduction is used to synthesise ATP molecules. Mutations in genes encoding subunits of the respiratory chain complexes can lead to impaired electron transport chain function, resulting in excessive production of reactive oxygen species (ROS) (15). Increased oxidative stress induces further DNA damage, thereby promoting genomic instability and the progression of neoplastic processes.

Homopolymeric regions are uninterrupted stretches of nucleic acids composed of repeated identical nucleotides. In the examined genes (*MT-CO1*, *MT-CO2*, and *MT-CO3*), a total of 25 such regions were identified, typically comprising short sequences of five or six identical nucleotides. Due to their structure, these sequences are particularly vulnerable to replication errors resulting from the DNA polymerase slippage mechanism, which leads to spontaneous nucleotide insertions or deletions (6). Indels in coding sequences most often cause a frame shift, leading to the synthesis of non-functional products or premature termination

of translation. For this reason, accurate detection of homopolymeric regions during sequencing is crucial for correct interpretation.

Oxford Nanopore Technology (ONT) is a third-generation DNA and RNA sequencing technique based on the passage of nucleic acid molecules through pores in a biological membrane and on the measurement of changes in electrical conductivity caused by interactions with individual nucleobases. Compared with earlier sequencing methods, ONT enables substantially longer sequence reads, averaging approximately 10 kilobases (kb) and reaching up to several hundred kb, to be generated in real time. ONT has particular advantages in the study of large genomes, such as the human or canine genome, in discovering the architecture of highly repetitive regions, detecting complex structural variants, and reconstructing haplotypes, although it also generates new computational challenges (14). Nevertheless, ONT is more prone than other technologies to sequencing errors, particularly in the case of homopolymeric sequences, therefore it requires much more careful data interpretation.

The aim of the study was to validate, by Sanger sequencing, ONT-detected insertion variants in homopolymeric regions of the *MT-CO1*, *MT-CO2* and *MT-CO3* genes, that had been identified by the Medaka algorithm as potential false positives.

Material and methods

A total of 22 mitochondrial DNA samples were analyzed from five dogs with primordial tumors and their recurrences. Eleven samples obtained from blood (n = 11) and tumor tissues (n = 11) were examined. Detailed information is provided in Table 1. The analyzed DNA was extracted from postoperative tumor tissue and from blood collected from of the examined dogs. Neither hormone therapy nor chemotherapy had been administered to the dogs. Recurrence and metastasis were assessed by histopathological examination and classified according to the WHO histological classification (3, 4). The collected samples were routinely fixed with 10% buffered formalin (pH 7.2), dehydrated through increasing concentrations of alcoholic solutions, cleared in acetone and xylene, and embedded in paraffin blocks. For histopathological analysis, sections were stained with haematoxylin and eosin and examined under a light microscope equipped with a digital camera (Olympus BX43, Olympus SC100, Tokyo, Japan) in accordance with WHO histological recommendations (International Classification of Tumours of Domestic Animals). The study was approved by the II Local Ethical Commission for animal experiments in Lublin, Poland (resolution number 6/2013).

Total DNA was isolated with NucleoSpin® DNA RapidLyse (MachereyNagel) according to the manufacturer's protocol. For blood samples, the standard protocol for fresh and frozen samples was used. For tumor samples, the protocol for challenging samples was followed, with additional mechanical lysis using glass beads. The amount of DNA obtained after the extraction ranged from 13 to 151 ng/µl. DNA quality control was performed by measuring the absorbance ratio at 260/280 and 260/230 nm. Samples below the threshold of 2.0 for A260/230 ratio and below 1.8 for A260/28 ratio were not included into the analysis. Mitochondrial DNA was amplified with two sets of primers specified in the literature (5). As a result, 9,653 bp and 9,942 bp overlapping fragments were obtained. Barcoded PCR products were sequenced with the LSK-109 library construction kit (Oxford Nanopore Technologies, Oxford, UK). Briefly, PCR products purified on magnetic beads were subjected to the end repair and dA-tailing procedure, followed by sequencing-adapter ligation. The library was sequenced on the R9.4.1 Flow Cell using a GridIONx5 device. Consensus sequences were generated from BAM files using the Medaka consensus algorithm (12). Reads were aligned to the NC_002008.4 reference genome (16,727 bp) using the Minimap2 algorithm (13). Alignments of reads to consensus sequences and of consensus sequences to the reference genome were also generated.

For reads aligned to the reference, variant call format (VCF) files were prepared using the Medaka variant algorithm. In addition, a VCF file was created for the alignment of consensus sequences to the reference genome using the SAMtools mpileup algorithm. Variants from both types of VCF files were intersected using the bcftools isec algorithm to obtain VCF files with variants between the consensus and the reference, with additional information on variant prevalence and prediction quality for a given haplotype, derived from read phases generated by the Medaka variant program. The VCF and BAM files

Tab. 1. Information regarding the samples analyzed and the dogs from which they were obtained

Dog breed	Sex	Age at diagnosis (age at recurrence)	Sample – Tissue
Labrador	Male	8	B25K – blood B25G – liposarcoma B40K – post-recurrence blood sample B40G – recurrent tumor
Crossbreed	Male	9 (10)	B47K – blood B47G – haemangiopericytoma B111K – post-recurrence blood sample B111G – recurrent tumor B180K – blood B180G – metastatic tumor
Bernese Mountain Dog	Female	8 (9)	B167K – blood B167G – mastocytoma B190K – blood B190G – mastocytoma
Crossbreed	Female	13	B166K – blood B166G – locally malignant haemanigoma B204K – post-recurrence blood B204G – recurrent tumor
Amstaff	Male	9	B162K – blood B162G – schwannoma malignum B169K – post-recurrence blood B169G – recurrent tumor

Tab. 2. List of indels in *MT-CO1*, *MT-CO2* and *MT-CO3* genes excluded from the analysis

Gene	Gene range	Position	Variant	Number of samples in which the variant occurred	Samples in which the variant occurred
MT-CO1	5349-6893	m.6023	m.6023insG	1	B25K
		m.6068	m.6068insC	3	B47G, B162K, B204G
		m.6625	m.6625insC	3	B25K, B25G, B40K
		m.6753	m.6753insT	1	B25G
MT-CO2	7034-7717	m.7073	m.7073insC	5	B47K, B47G, B111G, B180K, B180G
		m.7760	m.7760insT	2	B111G, B180G
MT-CO3	8644-9427	m.8896	m.8896insT	20	B25K, B25G, B40K, B40G, B47K, B47G, B111K, B111G B162K, B162G, B169K, B169G, B166K, B166G, B167K, B167G, B180G, B190K, B204K, B204G
		m.8916	m.8916insT	16	B25K, B25G, B40G, B47G, B111K, B162K, B169K, B169G, B166K, B167K, B167G, B180K, B180G, B190K, B204K, B204G
		m.9197	m.9197insT	6	B25K, B111K, B111G, B162G, B166K, B204G
		m.9231	m.9231insT	9	B40K, B47K, B47G, B111K, B162K, B166G, B169K, B190K B204K
		m.9299	m.9299insC	1	B180G

generated during the analysis were visualized in the Integrative Genomic Viewer (IGV) version 2.8.0 (19). The variant calls in the analyzed samples were determined according to the methodology used by Strakova et al. (17). If the read frequency for a variant was greater than 25%, the call was accepted.

ONT sequencing is particularly prone to insertion and deletion errors in homopolymeric regions due to the nature of nanopore translocation dynamics. To mitigate these errors, following steps were incorporated: 1. Bioinformatics tools were employed to identify variants located within homopolymeric regions. Variants detected in these regions were flagged as potential false positives. 2. Variants identified within homopolymeric regions were excluded from downstream analyses to prevent the inclusion of artifacts. 3. A list of indels in *MT-CO1*, *MT-CO2* and *MT-CO3* genes excluded from the analysis is provided in Table 2. This table details the specific indels identified as homopolymer-related artifacts, thereby ensuring transparency in the data processing. A list of all homopolymeric regions in *MT-CO1*, *MT-CO2* and *MT-CO3* genes is provided in Table 3. By integrating Medaka-based advanced error correction with targeted strategies for addressing homopolymer-induced errors, the accuracy of the mtDNA variant analysis was improved.

To verify whether the variants located in homopolymeric regions were false positives, Sanger sequencing was performed. Using the Primer3 program (11), primers were designed to flank positions considered false positives in the *MT-CO1*, *MT-CO2* and *MT-CO3* gene regions, that occurred in the largest number of samples. The primer sequences were as follows: F1 – 5'ACCCCTATATATCTCTATGGCGT3', R1 – 5'GCGGGTAGAATGGTTCATACTG3' flanking position m.7073 and F2 – 5'CGAGAAGGCACATTCCAAGG3', R2 – 5'CCCAAGTTCAGGAGTAGGGG3' – flanking positions m.8896 and m.8916. To amplify the *MT-CO1* gene region, primers COIF: 5'CCGGACATGGCATTCCCCCG3' and COIR: 5'GGCGGACGTAAAGTACGCTCGTG3' from Ślaska et al. (18) were used. Regions containing homopolymeric regions were amplified by polymerase chain reaction (PCR) using a Syngen Labcycler (Syngen Biotech, Wrocław, Poland). The annealing temperatures were 53.5°C for the region containing position m.7073, 55.9°C for the region containing positions m.8896 and m.8916 and 58.5°C for the *MT-CO1* region. PCR reac-

Tab. 3. List of homopolymeric regions in *MT-CO1*, *MT-CO2* and *MT-CO3* genes from ONT-derived sequences

Gene	Start	End	Sequence	Repeats	Detected indels
MT-CO1	5574	5578	GGGGG	5	–
	5630	5634	CCCCC	5	–
	5735	5740	CCCCCC	6	–
	5865	5869	CCCCC	5	–
	6003	6008	TTTTTT	6	–
	6023	6027	GGGGG	5	m.6023insG
	6068	6072	CCCCC	5	m.6068insC
	6138	6143	AAAAAA	6	–
	6192	6196	TTTTT	5	–
	6505	6509	TTTTT	5	–
	6625	6629	CCCCC	5	m.6625insC
	6753	6757	TTTTT	5	m.6753insT
	6843	6847	CCCCC	5	–
	6886	6890	AAAAA	5	–
MT-CO2	7073	7077	CCCCC	5	m.7073insC
	7543	7547	AAAAA	5	–
	7675	7679	CCCCC	5	–
	7760	7764	TTTTT	5	m.7760insT
MT-CO3	8870	8874	AAAAA	5	–
	8897	8901	TTTTT	5	m.8896insT
	8917	8921	TTTTT	5	m.8916insT
	8980	8984	GGGGG	5	–
	9197	9201	TTTTT	5	m.9197insT
	9231	9237	TTTTTT	6	m.9231insT
	9299	9303	CCCCC	5	m.9299insC

tions for all regions were performed in a total volume of 25 µl, consisting of 16.1 µl H_2O, 2.5 µl buffer, 2.5 µl dNTP mixture, 2.5 µl $MgCl_2$ solution, 0.15 µl of each primer pair and 1 µl of template DNA. PCR products were assessed qualitatively by electrophoretic separation in 1.5% agarose gel using BioStandard agarose (ABO, Poland), which is intended for routine electrophoretic separations. The amplicons were purified using ExoSAP-IT (Thermo Fisher Scientific, Waltham, Massachusetts, USA) reagent according to the manufacturer's protocol. The amplified regions were sequenced externally using the Sanger method. The obtained sequences were compared with sequences obtained using ONT with the Unipro UGENE v. 51.0 program, using the CLUSTALW algorithm to align them. Based on the number of nucleotides in homopolymeric regions, it was determined whether the variants identified by ONT were false positives.

Results and discussion

The key result of the study was the absence of insertions in homopolymeric regions of *MT-CO1*, *MT-CO2* and *MT-CO3* genes in both canine tumor tissues and blood controls. Although ONT sequencing suggested insertions at several *loci*, Sanger validation did not confirm any of these calls, indicating that they were technical artefacts rather than true biological variants. The *MT-CO1*, *MT-CO2* and *MT-CO3* genes are located in the canine mitochondrial genome at positions 5349-6893 (1545 bp), 7034-7717 (684 bp) and 8644-9427 (784 bp), respectively. Fourteen homopolymeric regions were identified within *MT-CO1*, of which four showed the presence of insertions when sequenced using the ONT method. In the *MT-CO2* gene, insertion variants were found in two out of four homopolymeric regions present there, while in *MT-CO3* – in four out of seven regions. Details are provided in Table 3. The dominant type of change was thymine insertion, which occurred six times; four cytosine insertions and one guanine insertion were also recorded, whereas no adenine insertions were found. The highest frequency of calls was observed in the *MT-CO3* gene, particularly at positions m.8896 (n = 20), m.8916 (n = 16) and m.9197 (n = 6). Analysis of the distribution of variants across samples showed the highest number of calls (n = 5) in samples B25K (blood from a Labrador with liposarcoma), B47G (a crossbreed dog with haemangiopericytoma) and B180G (a recurrence of the same tumor). Variants in homopolymeric regions were found in all analyzed samples, with no relationship between insertion frequency and tissue type. Importantly, Sanger verification did not confirm the presence of any of the detected changes in the 41 repetitive positions within examined homopolymeric regions. These regions included m.7073insC in the *MT-CO2* gene m.8896insT and m.8916insT in *MT-CO3* gene. No significant variants in homopolymeric regions of the *MT-CO1* gene were selected for Sanger verification. Sanger sequencing confirmed the artefactual nature of the insertions, attributable to the specific characteristics of nanopore technology.

This is the first study to verify the accuracy of the Medaka algorithm in sequencing mitochondrial cytochrome c oxidase subunit genes in dogs with cancer. Previous analyses of these genes in canine tumors revealed a diverse pattern of variation. In the *MT-CO1* gene, mainly polymorphic changes have been observed, such as synonymous transitions (e.g. m.5367C>T or m.5444T>C), which do not affect the protein structure and occur in both cancerous and healthy tissues (9, 18, 21). A different pattern was observed in the *MT-CO2* gene. While earlier reports associated defects in this gene mainly with myopathies or cardiomyopathy (20), studies on solid mammary carcinoma revealed a new, non-synonymous somatic mutation m.7308A>G. This mutation leads to the substitution of asparagine with serine (p.Asn92Ser), which may suggest its potential involvement in the process of carcinogenesis in this particular subtype of cancer, despite the absence of marked changes in protein stability *in silico*. In turn, missense polymorphisms, e.g., m.8807G>A (p.Cys55Tyr), have been identified in the *MT-CO3* gene; although they change the amino acid sequence, they are often not classified as pathogenic in isolation but may contribute to a genetic background that favors mitochondrial genome instability (21). Because of their potential deleterious consequences caused by frameshift mutations, indel mutations have not yet been reported in the research of genes encoding cytochrome c oxidase subunits in dogs.

No significant relationship was observed between gene length and the number of homopolymeric regions. The density of occurrence of these regions per 1,000 bp is 9.06 for the *MT-CO1* gene (1545 bp), 2.92 for the *MT-CO2* gene (684 bp) and 8.93 for the *MT-CO3* gene (784 bp). The homopolymeric sequence length was five nucleotides in 21 cases and six nucleotides in four cases; no longer regions were observed (Tab. 3). This result is consistent with the study of mononucleotide regions in prokaryotes by Coenye and Vandamme (1), according to which shorter repeats are substantially more numerous than longer repeats. Long mononucleotide repeats are generally absent in protein-coding regions. This is a protective mechanism that prevents frameshift mutations that could damage genes. Given the bacterial origin of mitochondria, these findings may suggest that the frequency and length of homopolymeric regions may depend on the importance of the gene in which they are present and on selective pressure to maintain the sequence integrity.

Despite its many advantages over other sequencing techniques, Oxford Nanopore Technology is particularly susceptible to generating indel errors in homopolymeric regions, as a direct result of the dynamics of DNA strand translocation through the nanopore. When a long sequence of identical bases passes through

the pore, the nucleotide composition within the pore does not change sufficiently despite strand movement, resulting in the generation of a flat and uniform electrical signal without the characteristic step changes that allow for precise differentiation between successive bases. In such situations, algorithms interpreting raw data must estimate the number of repeats of a given nucleotide solely on the basis of the duration of this stable signal, which carries a considerable risk of error because of the irregular and variable speed of DNA strand translocation through the membrane. Since molecular movement is not perfectly uniform over time, software may misinterpret faster strand translocation as a smaller number of bases, causing a false deletion, or slower strand translocation as a larger number of bases, resulting in a false insertion (2).

In this study, a total of 41 repetitive positions were identified in the *MT-CO2* and *MT-CO3* genes, at which ONT-called insertions were detected but not confirmed by Sanger sequencing. Particular attention should be paid to the scale of the phenomenon within the *MT-CO3* gene: at position m.8896, the call occurred in 20 samples out of 22 tested, and at position m.8916 in 16 out of 22 samples tested. However, these calls did not represent true variants in either canine tumor tissues or blood. According to Delahaye and Nicolas (2), basecalling depends not only on the bases currently present in the nanopore but also on the neighboring bases. If multiple identical bases are present in the vicinity of sequenced bases, an error in signal segmentation may occur and an incorrect read may be obtained in the context of the homopolymer sequence length. In the case of position m.8896C, the two preceding bases are thymine, which also constitute the homopolymeric region following this position. Similar situations occur in the case of position m.8916A, which is preceded by one thymine base, and at position m.7073C in *MT-CO2* gene, preceded by two cytosine bases. This fact may explain the much higher rate of false positive reads than in other regions.

Verification by Sanger sequencing clearly showed that none of the examined ONT-detected insertions was present in either tumor tissues or blood controls. Uncritical acceptance of these ONT calls would result in the misdiagnosis of indel mutations, which could lead to false conclusions based on the reading frame shift in a given sequence and the consequences thereof.

In order to ensure the reliability of data analysis obtained using ONT technology, it is necessary to apply appropriate error correction strategies, such as those applied by Kowal et al. (10), i.e. using dedicated algorithms such as Medaka, the use of bioinformatic tools for precise mapping of homopolymeric regions in the studied region and the exclusion of indel-type changes detected in these regions from statistical analysis.

This study shows, that distinction between biological reality and technical noise is crucial for a proper understanding of molecular biology, including the genetic background of canine cancers. Reliance on nanopore sequencing alone is insufficient for studying large genomes such as canine mitochondrial genome, as it carries a high risk of misidentifying technical errors as biological variants.

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Corresponding author: Dr Krzysztof Kowal; e-mail: krzysztof.kowal@up.lublin.pl