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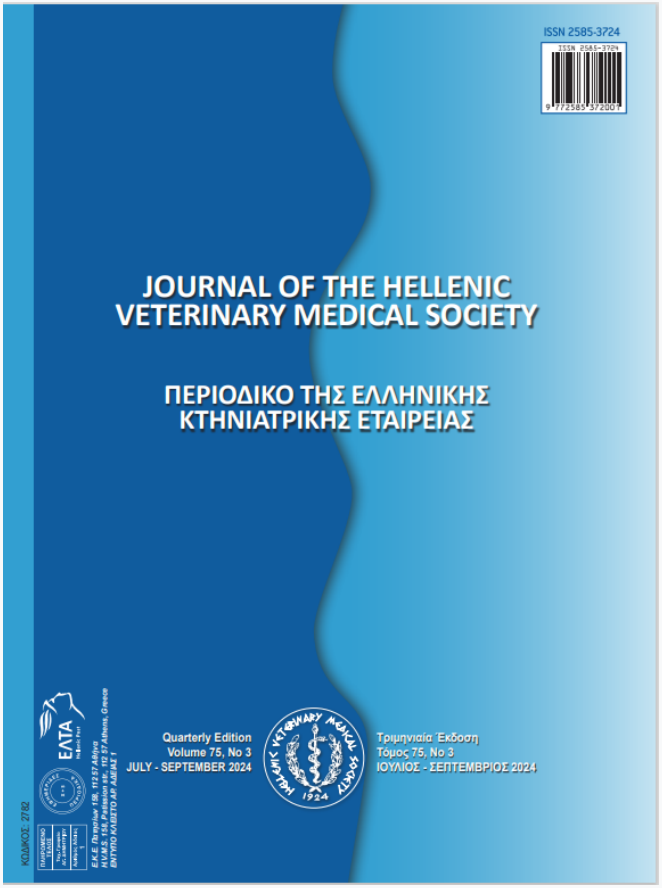

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Genetic changes clinically relevant in canine osteosarcoma

E. Lee, P. Loukopoulos

Melbourne Veterinary School, Faculty of Science, The University of Melbourne, Werribee, Victoria, Australia

ABSTRACT: Osteosarcoma is the most common bone tumour in dogs, and offers an excellent spontaneous model for its human counterpart. In this study, we reviewed the peer-reviewed literature on canine osteosarcoma using selected inclusion criteria, to identify studies on genetic changes that may be relevant to the pathogenesis or progression of canine osteosarcoma. A search of the CABI, Web of Science and PubMed databases initially identified 191 articles, 13 of which fulfilled the inclusion criteria. Genomic, transcriptomic and proteomic changes were identified, including in TP53, MMP-2, MMP-9, SETD2, DMD, HES1, NOTCH1, NOTCH2, HEY1, MET, MCL1, CDC5L, RUNX2, DAM15, and CTC1, employing methods including oaCGH analysis, whole genome sequencing, whole exome sequencing, RNA sequencing, cDNA microarrays, immunohistochemistry, RT-PCR, RT-qPCR, Western blotting, PCR, and *in silico* analysis. While not definitively established in all cases, the association of some of these changes with pathogenesis or clinically relevant parameters illustrated the potential of future studies employing similar methods to identify biomarkers of diagnostic, prognostic or therapeutic value.

Keywords: Dog; Genomics; Osteosarcoma; Transcriptomics.

Corresponding Author:
Panayiotis Loukopoulos, Melbourne Veterinary School, Faculty of Science, The University of Melbourne, Werribee, Victoria, Australia.
E-mail address: panos.loukopoulos@unimelb.edu.au

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INTRODUCTION

Osteosarcoma (OSA) is a malignant tumour of osteoblasts, forming neoplastic bone and/or osteoid (Loukopoulos et al., 2005). The cancer is most prevalent in children and canines, although occurring 10 times more frequently in the latter (Fenger et al., 2014). Up to 85% of malignant bone cancers are diagnosed as OSA, characterized by aggressive metastasis and rapid haematogenous dissemination, particularly to the lungs (Nelson and Couto, 2019, Thompson and Dittmer, 2020). These most commonly occur in dogs aged between 7-10 years (Selvarajah et al., 2009). The nature of canine osteosarcoma (CnOSA) gravitates to the appendicular skeleton, disproportionately affecting giant and large breeds, with 95% prevalence in dogs over 40kg, and 40% prevalence in dogs under 15kg (Nelson and Couto, 2019).

Regardless of the high prevalence of CnOSA, there has been little progression in improving survival rates in the last 50 years (Zapata et al., 2019). This may be a consequence of the spontaneity of the cancer in canines (Davis and Ostrander, 2014). However, recent combined utilisation of genomic/transcriptomic technologies has been of benefit to investigating genetic loci as causes and/or protectors against CnOSA (Davis and Ostrander, 2014). As a result, somatic mutations in the TP53, MYC, CDKN2A/B, PTEN, RUNX2, and DLG2 genes in humans and dogs with osteosarcoma- and KIT and MDM2 in OSA dogs alone have been identified (Zapata et al., 2019).

These findings are not only of prognostic importance to the several dog breeds with genetic predispositions to CnOSA (Simpson et al., 2017), but for humans too. Canine osteosarcoma has long served as an excellent spontaneous model of its human counterpart, because the two show close similarities regarding genetic changes (as shown above), clinical presentation and progression, and environmental factors. This is not purely for better diagnosis of at-risk patients, but for earlier management, and a foundation for novel approaches to treatment targets for OS.

Therefore, this literature review aims to identify the genetic changes clinically relevant in dogs with osteosarcoma.

METHODS

A search was conducted on Wednesday 20th April 2022 (see Figure 1). Databases searched include CABI, Web of Science and PubMed, using the

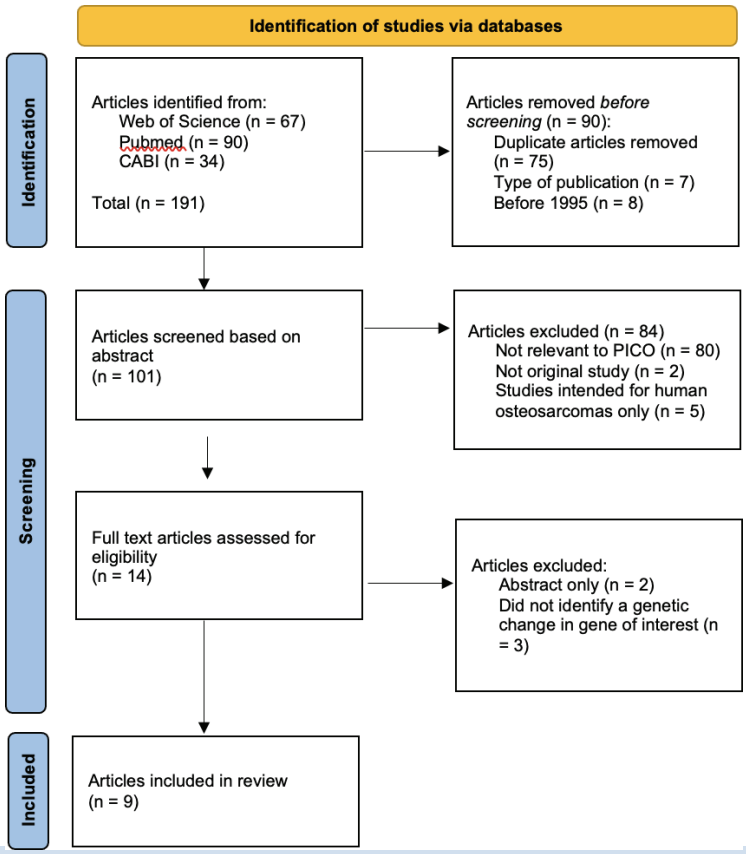

Figure 1. PRISMA flow chart for literature search performed

search terms (Canine OR dog) AND (osteosarcoma) AND (gene* OR genome) AND (change OR alteration). Nine papers were found using the inclusion and exclusion criteria mentioned below. Four further suitable journal articles were sourced and included in the review, totaling thirteen articles. Inclusion criteria consisted of any primary studies detailing changes in genes in dogs with osteosarcoma. Exclusion criteria consisted of the following: type of publication; published before 1995; based on canine models for study of human osteosarcomas only; not genomic/transcriptomic study; abstract only; no genetic change in gene of interest.

RESULTS

This review has been categorised into sampling, archival material, technologies used, genomics, proteins, and transcriptomics.

Sampling

Most studies followed an experimental study design, where CnOSA samples were compared to normal, non-neoplastic samples. Other studies chose to make comparisons between groups of disease-free intervals (DFIs) (Dailey et al., 2013; Dailey et al., 2021). This includes a study which explored cross-species genomics, comparing human OSA to CnOSA samples (Shao et al., 2019). Only two chose to study metastasis (Gardner et al., 2019; Lopez et al., 2018). The absence of normal cells as control populations produced a poor benchmark for identification of the genetic changes in CnOSA. However, comparisons of DFIs may aid understanding of the metastatic nature of the disease.

While the range of different sample categories provided a variety of overviews, it also created an inconsistency between findings.

Archival material

Kirpenstejin et al. (2008) observed TP53 gene mutations in 40% of CnOSA, consisting mainly of point mutations (74%). Dogs with mutations had a significantly shorter survival time after surgery than dogs with normal tumour TP53 gene expression. Use of archival material in the study by Sagartz et al. (1996) did not support a correlation between immunoreactivity and survival rates due to lack of clinical history. This could be considered a confounding factor for Kirpenstejin et al. (2008).

Methods used

Table 1 demonstrates the methods used in the studies reviewed and the corresponding molecular targets.

Variation in genomic and/or transcriptomic technologies created barriers in the synthesis of results, as each paper produced its own limitations, and thus little foundation for comparison.

Genomic studies identifying overexpression of

Table 1. Genomic and Transcriptomic Methods used

Study	Methods used	Target areas
Dailey *et al.* (2021)	RT-qPCR, cDNA microarrays	miR-103a, miR-223, Let-7b, miR-30c, miR-23a
Gardner *et al.* (2019)	Whole genome sequencing (WGS), whole exome sequencing (WES), RNA sequencing	TP53, SETD2, DMD
Lopez *et al.* (2018)	RNA isolation, RT-qPCR, cDNA synthesis	miR-34a
Leonardo *et al.* (2018)	In silico analysis, RT-PCR	miR-1, miR-133b, MET, MCL1
Fenger *et al.* (2016)	RNA isolation, cDNA synthesis, RT-PCR, RT-qPCR	Tissue- specific miRNAs - miR-9
Dailey *et al.* (2013)	Immunohistochemical analysis, RT-qPCR, Western blotting	HES1, NOTCH1, NOTCH2, HEY1
(Angstadt et al. 2012)	oaCGH analysis	CDC5L, RUNX2, DAM15, CTC1
Fossey *et al.* (2011)	RT-PCR, Western blotting	OSM
Kirpenstejin et al. (2008)	ImmunohBiopsy PCR	TP53
Loukopoulos et al. (2003)	Immunohistochemistry	P53
Loukopoulos et al. (2003)	Gelatin zymography	MMP2 and -9
Sagartz *et al.* (1996)	Indirect immunohistochemical method - immunohistochemistry and immunoelectron microscopy	P53

p53 tumour suppressor protein were based on a subjective scoring system (Sagartz et al., 1996). Confirmation of p53 positive tumours was based on a 0-3 scale of quality of immunohistochemical stain, subject to misclassification bias.

Transcriptomic studies mostly utilised RT-PCR, and RT-qPCR (Fenger *et al.* 2016). In some cases, repeatability by RT-qPCR was found to be low due to low miRNA yield, making it difficult to both control and identify poor-quality samples (Dailey et al., 2021). Low repeatability was also the case in a study by Lopez et al. (2018) in which tumour microenvironment and heterogeneity in tumour stroma and non-neoplastic infiltrates produced variations in miR-34a expression.

Despite the inconsistencies, these studies were still considered due to the progressive nature of the disease. CnOSA acts as a spontaneous canine disease, rapidly degenerating the bones of the affected (Davis and Ostrander, 2014). Appendicular skeletal OSA particularly metastasises to the lungs early in the disease, often before diagnosis of the primary tumour (Jubb et al., 2012). Therefore, inclusion of such studies may aid identification of CnOSA genetic changes regardless of the stage.

Genomics

TP53

The most common genetic mutations of CnOSA were revealed in the TP53 gene (Sagartz et al 1996). Gardner et al. (2019) reported TP53 gene mutations in CnOSA samples alongside novel mutations in SETD2 and DMD genes which affected tumour suppression function.

Matched normal samples and normal tissues from dogs with OSA showed no presence of TP53 gene mutations (Gardner et al., 2019; Kirpensteijn et al., 2008). Albeit normal tissues were not examined, Sagartz et al. (1996) found 84%, 56% and 40% of tumours overexpressed p53 tumour suppression protein in the appendicular skeleton, axial skeleton, and extra-skeletal sites respectfully. Conversely, Kirpenstejin et al. (2008) found TP53 gene mutations predominated the axial skeleton despite a markedly greater proportion of appendicular skeletal n=47) to axial skeletal OSA samples (n=11), thus, possibly indicating that the proportion of TP53 gene mutations in CnOSA may not be directly related to location.

Additionally, dogs with OSA and mutated TP53 had shorter median survival times after surgery compared to normal TP53 expression, suggesting a correlation between TP53 mutations and progression of CnOSA (Kirpensteijn et al., 2008).

Other genomic changes

Fossey et al. (2011) found OSM was expressed in all canine osteosarcoma samples but not canine osteoblasts (CnOb). They proposed OSM expression promotes tumour cell invasion via a cascade effect, thus contributing to the invasive and metastatic nature of osteosarcoma.

Shao et al. (2019) detected DLG2 deletions in 56% of CnOSA samples, concluding that this may lead to subsequent acceleration in CnOSA progression.

Genetic changes could therefore pose as markers for tumour expression, suppression, protectors, or even metastasis and thus further understanding of CnOSA behaviour (Fossey et al. 2011).

Proteins

Dailey et al. (2013) analysed 20 tumour samples immunohistochemically, grouped into good responders (DFI > 300 days), and poor responders (DFI < 100 days). They uncovered a 2.57-fold increase in HES1 expression in CnOSA cell lines compared to normal bone tissue (Dailey et al., 2013). Furthermore, HES1 overexpression had a 4.608-fold association with a longer DFI post limb amputation and chemotherapy (Dailey et al., 2013). Hence, elevated HES1 signalling in CnOSA was relatively reduced in poorer responders, without correlation to aggressiveness (Dailey et al., 2013). Dailey et al. (2013) also reported decreased NOTCH1 expression in DFI<100d group, and increased NOTCH2 and HEY1 expression irrespective of DFIs.

Matrix metalloproteinases (MMPs) are enzymes implicated in the degradation and remodeling of extracellular matrix, tumour invasion and metastasis. Loukopoulos et al (2003, 2004) used gelatin zymography and immunohistochemistry to determine whether MMPs are present in canine tumours and normal tissues and whether MMP production correlates with clinicopathologic parameters of prognostic importance. High levels of pro-MMP-9, pro-MMP-2, and active MMP-2 were detected in most canine tumours. Significantly higher MMP levels were measured in canine tumours than in nontumours, malignancies had

higher MMP levels than benign tumours, and sarcomas had higher active MMP-2 than carcinomas. Cartilaginous tumors produced higher MMP levels than did nonsarcomatous malignancies, benign tumours, and normal tissues, and significantly greater MMP-2 than osteosarcomas and fibrosarcomas. Pro-MMP-9 production correlated with the histologic grade of osteosarcomas. The authors concluded that zymography proved to be a sensitive and quantitative technique for the assessment of MMP presence but has the limitation of requiring fresh tissue; immunohistochemistry is qualitative and comparatively insensitive but could be of value in archival studies.

Transcriptomics

Four transcriptomic studies reported changes in CnOSA microRNA expression (Fenger et al. 2016).

Fenger et al. (2016) identified significant overexpression of miR-9 in CnOSA tumours. miR-9 was sampled directly from malignant osteoblasts of primary tumours. Whilst miR- 9 expression was found to have no effects on cell proliferation or apoptosis in malignant cell types, inhibition of the microRNA did decrease cell invasion and migration in OSA cells (Fenger et al., 2016). Hence, it was proposed that overexpressed miR-9 contributed to the aggressive biological behaviour of CnOSA (Fenger et al., 2016).

Lopez et al. (2018) showed decreased expression of miR-34a in CnOSA compared to normal canine bone. Under-expression was found to promote cellular invasion and migration, and therefore subsequent metastasis (Lopez et al., 2018); further findings suggested miR-34a could be a target for management options, reducing rate of progression to metastasis.

Leonardo et al. (2018) reported that reduced expression of miR-1, miR-133b is associated with successively increased expression of anti-apoptotic and promotor genes responsible for tumour growth via a cascading pathway.

Dailey et al. (2021) showed that 19 miRNAs are overexpressed in tumours, and that increased miR-223a and decreased expression of miR-103a and Let-7b correlated with higher risk of recurrence in osteosarcoma. They also produced a two-miRNA model (miR-23a-3p and miR-30c-5p) which could allow for objective categorisation of CnOSA into prognostic groups; this could prove be beneficial for more accurate diagnosis and management based on estimated prognosis.

Understandably, involvement of genomic and transcriptomic technologies in research of canine oncology is still early in its development (Harrison and Loukopoulos, 2021). Therefore, further research is required to understand the effects of the changes in genes of OS, especially as technologies progress in efficacy.

CONCLUSION

Studies and discoveries of gene mutations have noted both genomic and transcriptomic alteration in CnOSAs compared to healthy dogs. Mutations in TP53, SETD2, DMD and HES1, and changes in expression of various microRNAs provide a foundation for understanding the behaviour of CnOSA, and therefore implications on outcomes. With access to genetic technology, there is ample opportunity to further investigate these genetic changes clinically relevant to CnOSA. However, there is no assurance of a single technique that could identify one genetic mutation more important than another. Development of new technologies, and further studies can ultimately aid identification of novel targets for diagnostic and therapeutic purposes. Regardless of the technologies, future studies may find more value in analysing change in CnOSA by making comparisons to a control group of normal CnOb or cell lines. This would create a benchmark for said changes to be compared to, thus establishing the cause-and-effect relationship. Furthermore, studies may find inclusion of metastatic samples beneficial, as it is an important factor of CnOSA. Genetic research can be of benefit to the population of dogs and humans who may be predisposed to osteosarcoma. Ultimately, valuable developments in technology could lead to identification of prognostic biomarkers for metastatic behaviour of the tumour.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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