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Genomic profiling of cell-free DNA from dogs with benign and malignant tumors

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Abstract

Objective Cancer is currently the most common cause of death in adult dogs. Like humans, dogs have a one-third chance of developing cancer in their lifetime. We used shallow whole-genome sequencing (sWGS) to analyze blood cell-free DNA (cfDNA) from four tumor-bearing dogs (one with benign and three with malignant tumors) and 38 healthy dogs.

Results Similar to the results observed in the healthy dogs, no copy number aberration (CNA) was detected in the dog with benign lipomas, and the distribution of cfDNA fragment size (FS) closely resembled that of the healthy dogs. However, among the three dogs diagnosed with malignant tumors, two dogs exhibited varying degrees and quantities of CNAs. Compared to the distribution of FS in the healthy dogs, the cancer dogs exhibited a noticeable shift towards shorter lengths. These findings indicated that CNA and FS profiles derived from sWGS data can be used for non-invasive cancer detection in dogs.

Keywords Dog, Liquid biopsy, cfDNA, CNA, FS, sWGS

Introduction

Cancer is the leading cause of death in dogs, primarily due to most cases are diagnosed at an advanced stage, resulting in a poor prognosis [1]. Growing evidence of cancer as a genomic disease has led to the emergence of liquid biopsy. Liquid biopsy is widely used for various applications, including screening, auxiliary diagnosis, targeted therapy selection, treatment response monitoring, minimal residual disease detection, and recurrence monitoring [2]. It broadly refers to the collection and analysis of various body fluid samples (mainly blood, occasionally urine, cerebrospinal fluid, or other secretions) using minimally invasive or non-invasive methods. Blood-based liquid biopsy tests involve analyzing circulating tumor cells (CTCs), proteins, and circulating tumor DNA (ctDNA) from cancer patients. CTCs can be technically more difficult to isolate than ctDNA [3]. Protein markers have been used for cancer screening, such as PSA for prostate cancer. However, they have not been established for canine cancer early detection. ctDNA consists of DNA fragments released into circulation by tumor cells through secretion, apoptosis, or necrosis. ctDNA is the most commonly employed method for liquid biopsy and is effective for multi-cancer

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early detection in asymptomatic populations [4, 5]. Recently, more studies have also demonstrated the presence of ctDNA in canine plasma across multiple cancer types, highlighting its potential for early diagnosis and therapeutic intervention in canines [6–8]. Copy number aberrations (CNAs) are prevalent across various types of cancer [9]. Similar to tissue samples, ctDNA can be used to detect tumor-induced genomic alterations, including CNAs. This method has been effectively utilized for cancer detection in both humans and dogs [10, 11]. Moreover, it is well established that the fragment size (FS) of ctDNA in human cancer patients is typically shorter than that of cfDNA in healthy individuals, which can increase the sensitivity of cancer detection [12, 13]. Here, we performed sWGS analysis on 42 dogs, one with benign masses, three with malignant masses, and 38 healthy dogs, to evaluate differences in CNA and FS data and to demonstrate that cfDNA-based sWGS analysis could be used for dog cancer detection.

Main text

Methods

Materials

Four dogs bearing tumors were enrolled in this study, and informed consent was obtained from the owners. One tube of blood from each dog was collected using 5 ml Cell-Free DNA Blood Collection Tubes (Ardent BioMed, Guangzhou, China) by licensed veterinarians from two veterinary hospitals: New Ruipeng Pet Healthcare Group Co. (Beijing, China) and Shanghai Companion Animal Hospital (Shanghai, China).

Additionally, the sWGS data from the 38 healthy dogs with no history of cancer or related symptoms were obtained from our previous study and used as controls [13]. The experimental procedures were identical to those applied to the four tumor cases.

sWGS

cfDNA was subsequently extracted from the blood using the QIAamp Circulating Nucleic Acid Kit (QIAGEN, Dusseldorf, Germany) following the manufacturer's instructions. Subsequently, the cfDNA was utilized for library construction with the Kapa Hyper Prep Kit (Kapa Biosystems, Wilmington, USA) as per the manufacturer's instructions. The resulting libraries were sequenced on a NovaSeq system (Illumina, San Diego, USA) to generate paired-end 150 bp reads, resulting in approximately 3 Gb (~ 1x coverage) of raw data. After removing adapters and eliminating low-quality reads, the clean reads were aligned to the dog genome (https://hgdownload.soe.ucsc.edu/goldenPath/icanFam3/bigZips/).

CNA analysis

For CNA detection, a well-established methodology from our previous study was employed [9]. In brief, the dog genome was divided into non-overlapping 1 Mb bins, and read counts were tabulated, followed by GC correction. Read counts were then normalized using a cohort of 38 healthy dog samples from our previous study [13]. The normalization process yielded Z-scores, calculated by subtracting the mean value of the healthy sample dataset and dividing by the standard deviation. These Z-scores were then input into a circular segmentation algorithm, as provided by the R package "DNAcopy", to make segmentation calls. Whole genome copy number changes were used to compute the chromosomal instability (CIN) score, determined by summing the Z-scores of all segments, each multiplied by its respective length. $CIN\_Score = \sum V_{segment} \times L_{segment}$, where V represents the Z-score of a segment and L signifies its length.

FS analysis

FS analysis was performed based on the sWGS data [9]. Specifically, the size of cfDNA fragments was calculated based on the mapping position of the remaining paired-end reads. The number of cfDNA fragments within each size range was aggregated, and the distribution of cfDNA FSs was determined. The short fragment ratio, denoted as P150, was defined as the proportion of cfDNA fragments falling within the 50 ~ 150 bp range.

Statistical analysis

All statistical analyses, including Gaussian distribution analysis and outlier analysis, were performed using R (v4.2.1).

Results

One beagle was randomly selected from the 38 healthy dogs as a demonstration case (Case 1). The CNA analysis revealed no amplifications or deletions across any of the 38 chromosomes. The CIN score of Case 1 was 205.6 (Fig. 1a). The cfDNA fragmentation profiles displayed peaks at 50, 60, 70, 81, 92, 102, 112, 122, 133, 143, 153, and 164 bp, consistent with the peaks observed in all healthy dogs (Fig. 1b). The P150 value for Case 1 was 35.9. The gray region represents the first and third quartiles of each FS proportion in all 38 healthy dogs. Both CNA and FS results fell within the comparison range of normal dogs, indicating the absence of a cancer signal in the blood of Case 1.

Case 2, a 5-year-old male Corgi, had a sagging mass on the left abdomen. The CNA analysis revealed no amplifications or deletions across any of the 38 chromosomes, and the CIN score was 203.5 (Fig. 1c). Like those of healthy dogs, the cfDNA fragmentation profiles displayed peaks at positions 50, 60, 70, 81, 92, 102, 112, 122,

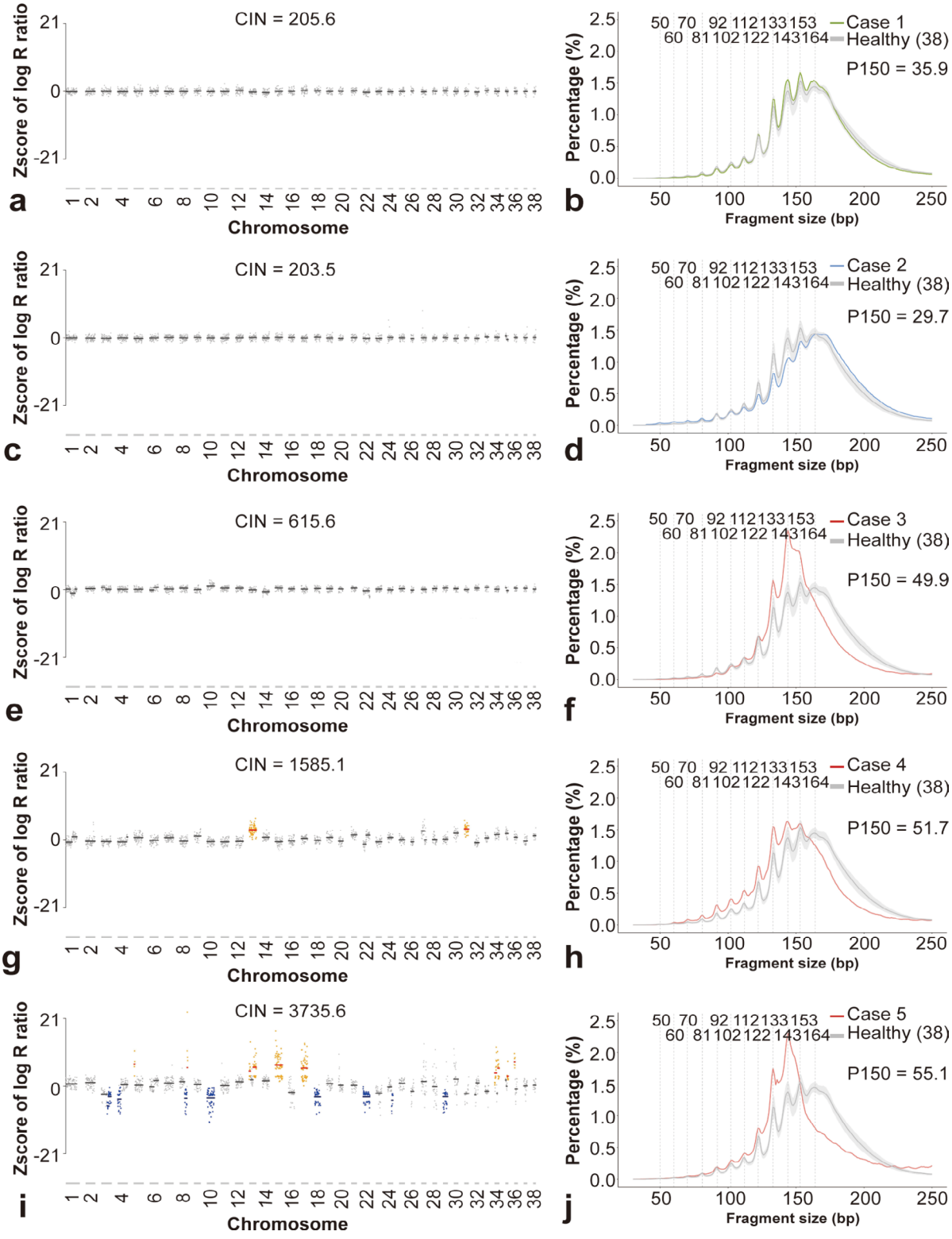

Fig. 1 sWGS analysis results of cfDNA from 5 dog cases. **a**, **c**, **e**, **g** and **i** displayed the CNA profiles of cases 1–5, respectively. Amplifications were represented by orange, deletions were represented by blue, and neutral segments were represented by gray. The X-axis represented the ID of chromosomes, and the Y-axis represented the Z-score standardization based on the log R ratio compared to the healthy controls. **b**, **d**, **f**, **h** and **j** displayed the FS distributions of cases 1–5. The gray region represented the first and third quartile of the FS distribution among 38 healthy dog blood samples. CNA, copy number aberration; CIN, chromosomal instability; FS, fragment size

133, 143, 153, and 164 bp. Notably, the dominant peak of Case 2 was at 164 bp, which was slightly longer than that observed in healthy dogs (Fig. 1d). The P150 value for Case 2 was 29.7, which falls within the range of healthy dogs (range: 25.2–44.5). Both the CNA and FS results indicated that the absence of a cancer signal in the blood from Case 2. The surgically excised tissue was assessed histologically. Based on the examination results, Case 2 was diagnosed with a lipoma, a benign tumor, consistent with the sWGS analysis. Lipomas are benign neoplasms comprising focal fatty nodules derived from adipocytes in the subcutaneous tissue [14]. In general, the prognosis after surgical resection is good [15]. Follow-up results also showed that the dog remained alive and well 15 months after diagnosis, with no complementary treatment other than surgery.

Case 3, a 14-year-old male Schnauzer, had a mass in the oral cavity. While no apparent CNAs were detected, the CIN score was 615.6, exceeding the maximum CIN score of all healthy dogs, which was 594.3 (Fig. 1e). None of its chromosomes had significant degrees of amplification or deletion (Fig. 1e). Furthermore, the cfDNA fragmentation profile indicated a shift towards shorter FS compared to healthy dogs and Case 2, with a dominant peak at 143 bp and a P150 of 49.9 (Fig. 1f). This finding suggested the detection of cancer signals in Case 3. It is a well-known fact that in humans, ctDNAs exhibit shorter fragment sizes than normal cfDNAs, and the proportion of short ctDNA fragments in plasma is positively correlated with the tumor DNA fraction [16]. In addition, the surgically excised tissue was assessed cytologically. Based on the examination results, Case 3 was diagnosed with an oral melanoma, a common malignant tumor in dogs [17]. The dog owner did not choose to pursue further examination or treatment for the dog, and euthanasia was performed on the dog 10 days later.

Case 4, a 6-year-old female Labrador, had a size of 10.0×8.3 cm space-occupying mass in the abdomen. The CNA analysis revealed varying degrees of amplification on chromosomes 13 and 31, along with a high CIN score of 1585.1, approximately five times the median of healthy dogs (median: 289.8; Fig. 1g), indicating the presence of cancer signals in the blood. Similar to Case 3, the cfDNA fragmentation profile displayed a shift towards shorter fragment lengths, with a dominant peak at 143 bp and a P150 of 51.7 (Fig. 1h). The surgically excised tissue was assessed histologically. Based on the examination results, Case 4 was diagnosed with a hemangiosarcoma, a highly aggressive and malignant tumor that originates from the cells lining blood vessels, with a poor prognosis and a high rate of metastasis [18]. Despite undergoing surgery and doxorubicin chemotherapy, splenic metastasis occurred four months post-surgery.

Case 5, a 12-year-old male Teddy, was presented with osteolysis of the stifle joint in the left hind limb. According to the sWGS analysis, the genome of Case 5 was quite different from those of the healthy dogs. Approximately 40% (15/38) of the chromosomes had different degrees of amplification or deletion. Among them, chromosomes 5, 8, 13, 15, 17, 34, 35 and 36 were amplified, and chromosomes 3, 4, 8, 10, 18, 22, 24 and 29 were deleted. Its CIN score, 3735.6, was extremely high, about 13 times the median of healthy dogs (median: 289.8; Fig. 1i). Additionally, the cfDNA fragmentation profile demonstrated a higher proportion of shorter fragments, with a dominant peak at 143 bp and a P150 of 55.1(Fig. 1j), indicating the clear detection of cancer signals. The surgically excised tissue was assessed cytologically. Based on the examination results, osteosarcoma, the most common primary bone tumor found in dogs, accounting for 80–90% of all malignancies originating in the skeleton, was diagnosed in Case 5 [19, 20]. This dog's condition deteriorated further four months after the initial visit, with osteolysis spreading to the femur of the left hind limb, and he was euthanized one month later.

We performed a comparative analysis of CIN scores and P150 values between 38 healthy dogs and four tumor-bearing dogs. A boxplot analysis was conducted using data from the healthy control group. CIN scores from the three cancer dogs (615.6, 1585.1, and 3735.6, respectively) were identified as outliers, with values higher than those of the healthy controls (Fig. 2a). Additionally, a Gaussian distribution analysis with a significance level of $\alpha = 0.05$ was applied to establish a statistical cutoff value based on the CIN scores of all 38 healthy dogs. CIN scores of the three cancer dogs, as well as three healthy dogs (573.8, 582.3, and 594.3, respectively), were found to be statistically higher than the upper cutoff value of 549.2. Gaussian distribution analyses indicated that the CIN score of Case 2, with a benign tumor, fell within the normal range of the healthy dogs, which was consistent with the result observed for P150 as well (Fig. 2b). Moreover, the P150 values of the three cancer dogs were 49.9, 51.7, and 55.1, respectively. These values were identified as outliers in the boxplot analysis based on the P150 values of the 38 healthy dogs and were also significantly higher than those of the healthy dogs according to Gaussian distribution analysis.

Discussion

With the advancement of sequencing technology, liquid biopsy has been widely used in cancer diagnosis and monitoring [21]. In this study, we used sWGS to detect cancer-related signals in the bloodstream of 42 dogs. CNAs were detected in two out of three cancer dogs. A previous human cancer study had shown that the incidence of CNAs in non-central nervous system solid

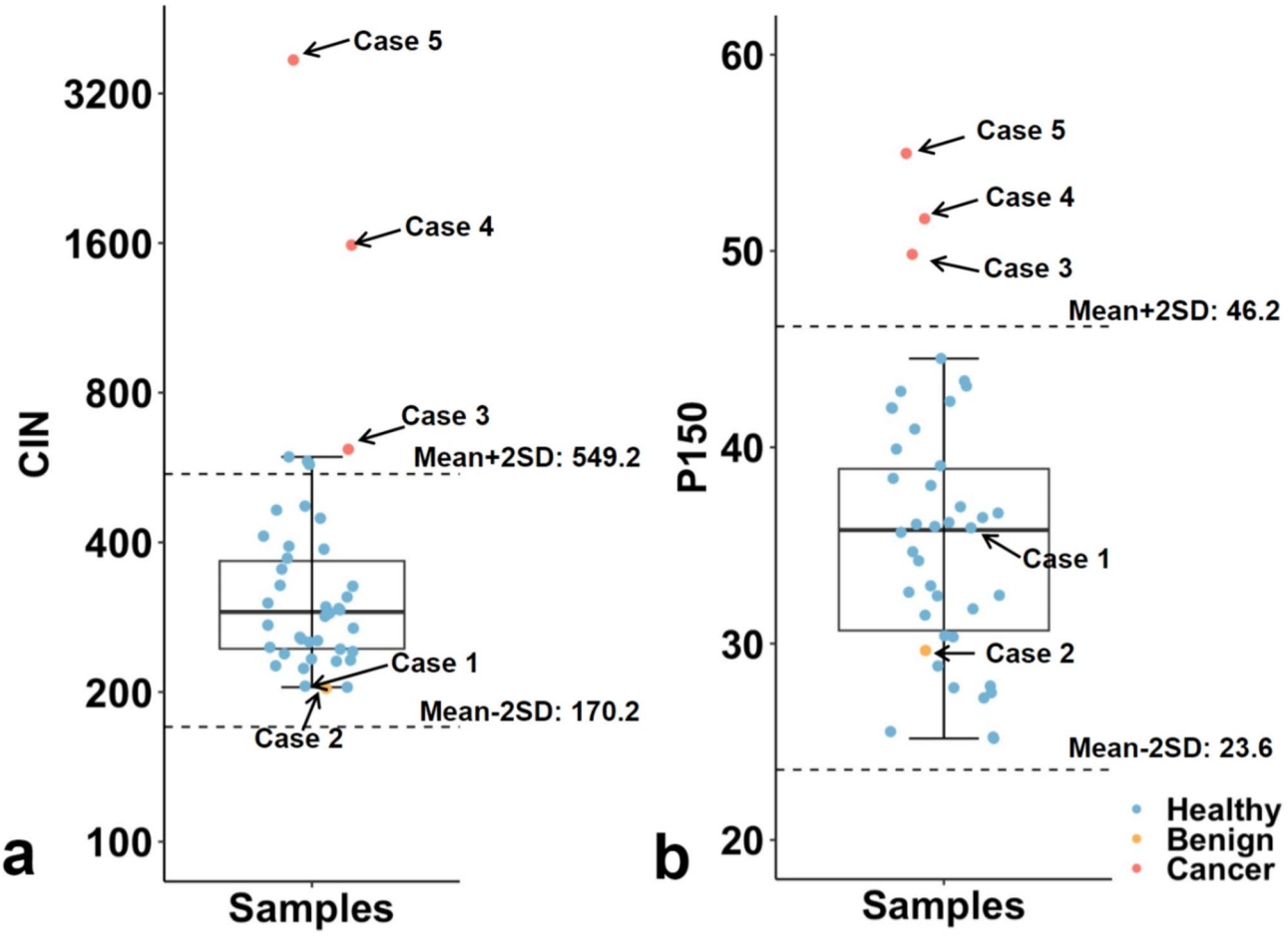

Fig. 2 Comparative analysis of cases ($n=4$) and controls ($n=38$). Three dogs with malignant tumor were represented in red dots, a benign tumor dog was represented in an orange dot, and 38 healthy dogs were represented in blue dots. The dashed lines represent the established statistical cutoffs (significance level: $\alpha=0.05$) based on the CIN and P150 scores of all healthy dogs. **(a)** Displayed the CIN scores of 4 tumor cases and 38 healthy dogs. The Y-axis is plotted on a logarithmic scale to accommodate the wide range of CIN values. **(b)** Displayed the P150 for 4 tumor cases and 38 healthy dogs. CIN, chromosomal instability; P150, proportion of cfDNA fragments falling within the 50 ~ 150 bp range

tumors was approximately 70.6% [22]. This finding suggests that CNA can serve as a genomic feature for the early detection of malignant cancers in dogs, its utility may be enhanced when combined with other cancer genomic features, such as the FS. Furthermore, the absence of detectable CNAs in all healthy dogs reinforces the notion that CNA is a cancer-specific feature, as previously established in human genomic studies [23].

Additionally, the proportion of shorter FS fragments (P150) in the three cancer dogs was significantly higher than that observed in the healthy dogs, consistent with the FS profiles commonly reported in human cancers. The FS feature has been widely utilized for the early detection of various cancer types [10, 24]. Our previous study demonstrated that cfDNA fragmentation occurs through a stepwise process, both inside and outside apoptotic cells, and is conserved across mammals, including canines [13]. Therefore, despite the limited sample size of cancer dogs, our data further support the potential application of FS as a feature for early cancer detection in dogs, especially when combined with CNA via sWGS.

Although there are only three cancer dogs in the study, we have observed potentially different fragmentation profiles among them. For example, Cases 3 and 5 have a prominent peak at 143 bp, while the higher proportion of short fragments in Case 4 are more evenly distributed. Due to small sample size, we will not be able to relate it to breed or tumor stage. Larger studies may have the statistical power to analyze the distribution of short fragments and be able to address the other features of cfDNA like end motifs in cancer dogs in the future [13].

The degrees of genomic abnormalities in the three malignant tumor cases varied, which was consistent with the tumor malignancy determined by tumor type and stage [25, 26]. This correlation highlights the relationship between CIN scores, FS values, and tumor burden.

The Gaussian distribution statistical analysis revealed that the CIN score and P150 value of the dog with a benign tumor (Case 2) fell within the normal range of the healthy control group. This suggests that our test could serve as a complementary tool for distinguishing between benign and malignant tissues.

Our study demonstrated the potential of sWGS-based liquid biopsy for detecting cancer signals in dogs. These findings emphasize the feasibility of utilizing cfDNA analysis for non-invasive cancer screening and diagnosis in canine patients. Early detection through liquid biopsy can facilitate timely intervention and expand treatment options. Moreover, this approach has the potential to become a more affordable testing option for pet owners, thereby enhancing access to advanced cancer diagnostic tools.

Limitations

1. The number of cases was too small. Further validation with additional cases is warranted to confirm the efficacy of sWGS-based cfDNA analysis in detecting cancer in dogs.
2. Due to the lack of consent from the dog owners for a postmortem examination, only the pathological results of the patients' tumor tissue biopsies were currently available. Future observed cases will require additional clinical diagnostic information, such as postmortem results and tumor typing results.

Abbreviations

sWGS	Shallow whole-genome sequencing
cfDNA	Cell-free DNA
CNA	Copy number aberration
FS	Fragment size
CTC	Circulating tumor cell
ctDNA	Circulating tumor DNA
CIN	Chromosomal instability

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Author contributions

M.M. and S.L. designed the study; H.D., W.L., and P.H. collected participants' samples and clinical information; D.Z., J.L., and N.Y. performed experiments; S.L. and Y.L. designed bioinformatics pipelines and analyzed results; H.D., W.L., and P.H. interpreted the pathological examination data. L.Z. and F.J. wrote the manuscript; S.L., M.M., H.D., and W.L. revised the manuscript. All authors read and approved the final manuscript.

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Data availability

Sequencing data of the 38 healthy dogs and 4 tumor cases, as well as clinical information about these dogs are available with open access at https://ngdc.cncb.ac.cn/bioproject/browse/PRJCA029622.

Declarations

Ethics approval and consent to participate

No experimental studies were conducted on the dogs, and dogs were not experimental. According to Chap. 1, Article 2 of document No. 167 (2023) issued by the National Science and Technology Administration (on September 7, 2023), "Regulations on Science and Technology Ethical Review," this research did not require official or institutional ethics approval. Informed consent for the participation of the dogs was obtained from their owners.

Consent for publication

Not applicable.

Competing interests

P.H. is a full-time employee of and holds stock options in TwixBio. Y.L., D.Z., M.M., and S.L. hold stock options in TwixBio. All other authors declare no competing interest.

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