Quantification of plasma cell-free DNA levels in dogs with various tumors

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Running head: cfDNA in dogs with various tumors

Abstract. Circulating cell-free DNA (cfDNA) is extracellular DNA released into the blood stream by apoptotic or necrotic tumor cells, with cfDNA determination proposed as a non-invasive, sensitive marker for the diagnosis of human cancer. We evaluated cfDNA quantification as a diagnostic and prognostic tool in dogs with various tumors. We quantified plasma cfDNA concentration by absolute real-time polymerase chain reaction of long interspersed nuclear elements in 50 dogs with malignant tumors, 13 dogs with benign tumors or nodules, and 11 healthy controls. Six patients with malignant tumors were followed-up, and plasma cfDNA was quantified throughout disease progression. We found that plasma cfDNA concentrations were significantly elevated in dogs with malignant tumors compared with dogs with benign nodules or healthy controls. The DNA integrity index was significantly lower in dogs with malignant tumors compared to healthy controls. Significantly higher cfDNA levels and a lower DNA integrity index were observed in dogs with lymphoma or leukemia, hemangiosarcoma, and distant metastasis; cfDNA levels correlated well with clinical stage and tended to increase during or before periods of disease progression, suggesting potential efficacy of cfDNA for the detection of distant metastasis and to monitor the clinical stage of neoplasia.

Key words: cell-free DNA; dogs; liquid biopsy; tumor biomarker.

Introduction

Various tumors occur naturally in dogs and cats, with cancer being among the most common causes of death in these animals. Veterinary oncology is required to provide early and effective cancer detection.[14] Tumor biomarkers have been used in human medicine to predict the outcome of therapy response and prognosis,[2,16,17] and such markers would be useful in the detection and monitoring of neoplasia in animals. Biomarkers, including their differential expression or mutation in microRNAs, circulating tumor DNAs, proteins, exosomes, and circulating tumor cells, are noninvasive means to identify the status of tumor development.[16,28] However, available tumor biomarkers are limited in veterinary medicine.

Circulating cell-free DNA (cfDNA) is extracellular DNA released into the bloodstream as a result of apoptosis, necrosis, and secretion.[10] Another important source of circulating tumor DNA is exosomes, which are cell-released membranous structures that contain proteins, lipids, and nucleic acids.[5] Low levels of cfDNA can be detected in healthy individuals; however, higher levels have been detected in human patients with a number of diseases, including cancer.[3,25] Changes in plasma cfDNA levels are reflective of tumor burden, with cfDNA methylation, cancer-derived mutation, and loss of heterozygosity representing potential biomarkers useful for cancer detection.[34] Compared with traditional tissue biopsy, analysis of plasma cfDNA has obvious advantages and provides a rapid, cost-effective, and noninvasive “liquid biopsy” method capable of potentially providing important complementary diagnostic information concerning therapeutic targets and drug-resistance

mechanisms in cancer patients.[33] cfDNA concentration has been quantified using various techniques, including UV spectrophotometry, fluorescence-based nucleic acid quantification, and quantitative polymerase chain reaction (qPCR).[22] A study using dogs with sepsis, trauma, and neoplasia demonstrated excellent linearity between UV spectroscopy and fluorescence assays for cfDNA measurement.[15] Regarding cfDNA, the 180-bp fragment reflects apoptosis, which is the prevalent mechanism associated with normal cell death, whereas necrosis produces longer DNA fragments and occurs more frequently in tumor cells.[10]The degree of cfDNA integrity (i.e., the integrity index) is the ratio between long and short cfDNA fragments.[3] The DNA integrity index was recently proposed as a promising specific oncologic biomarker given its high degree of sensitivity.[3,4,9]

Increased blood cfDNA concentrations have been documented in dogs with immune-mediated hemolytic anemia, cancer, sepsis, gastric dilatation–volvulus syndrome, and trauma[1,11,15,29]; however, the veterinary cancer literature in this area is limited. Therefore, we assessed the value of plasma cfDNA samples using long interspersed nuclear element-1 (LINE-1) quantification as a diagnostic marker and predictor of metastasis in dogs with various tumors.

Material and methods

Sample collection and processing

Blood samples from 63 dogs with various tumors and 11 healthy controls were collected at the Veterinary Medical Center at the Obihiro University of Agriculture and Veterinary

Medicine (VMC-OUAVM) between January 2018 and January 2019. Our study was approved by the Institutional Animal Care and Use Committees at OUAVM (permission 18-2).

The neoplasia group comprised 50 dogs with malignancies and 13 dogs with benign tumors or nodules, with all of the diagnoses confirmed histologically or cytologically at the VMC-OUAVM. Staging of the malignancies was performed according to the World Health Organization staging system,[21] including the use of computed tomography, 3-view thoracic radiography, abdominal ultrasound, and hematologic examinations. Six patients with malignant tumors underwent multiple blood collections over time in order to determine whether cfDNA levels correlated with disease progression (Table 2). Definitions for the response to treatment and relapse criteria used at each visit were those described for lymphomas[30] and other solid tumors[20] based on physical examination and additional tests at the discretion of the clinician. Dogs in the control group were healthy patients arriving at the hospital for medical examinations. Two mL of peripheral blood was collected in ethylenediaminetetraacetic acid from each dog prior to biopsy or surgical excision; plasma was separated by centrifugation at 2000 × *g* for 10 min at 4°C, transferred to new tubes, and centrifuged at 16,000 × *g* for 10 min at 4°C to remove cell debris. Plasma was stored at −30°C prior to DNA extraction, and all samples were processed within 4 h of blood collection.

cfDNA extraction and quantification

cfDNA was isolated from 500 µL of plasma (MagMAX cell-free DNA isolation kit; Qiagen, Valencia, CA) according to the manufacturer's instructions. cfDNA preparations were eluted

93 in 50 µL of elution buffer and stored at −30°C until further analysis.

94 Plasma cfDNA concentrations were measured by qPCR in order to quantify short and
95 long cfDNA fragments. To maximize quantification sensitivity, the LINE-1 retroposon
96 (GenBank accession AY266086.1), which is the most abundant repeat sequence present in the
97 canine genome,[31] was used as a qPCR target. Two primer pairs were used as follows: LINE-
98 99 primers amplified both the short (99 bp) and long (218 bp) fragments, and LINE-218
99 primers amplified only the long DNA fragments. The results obtained using the LINE-99
100 primers represent the total free plasma DNA, whereas results obtained using the LINE-218
101 primers reflect the amount of DNA released from non-apoptotic cells. The quantitative values
102 from the 99-bp amplicon represent total cfDNA concentration (ng/mL), whereas the ratio of
103 long to short fragments represented the "DNA integrity index" in each plasma sample.[1]
104 Primer sequences were as follows: LINE-99 forward, 5′-AAATGCAATGAAACGCCGGG-3′;
105 and LINE-218 forward, 5′-TGGGAATGTGAACTGGTGCA-3′ and reverse, 5′-
106 TCTTTCGTTGGACACCGAGG-3′.[15] The qPCR reaction was performed in a 20-µL reaction
107 volume containing 500 nM of each primer, 1 µL of cfDNA template, and 10 µL of PowerUp
108 SYBR Green master mix (ABI; Thermo Fisher Scientific, Waltham, MA) and using a
109 StepOne Real-Time PCR system (ABI; Thermo Fisher Scientific). Cycling was as follows:
110 initial incubation at 50°C for 2 min and 95°C for 2 min, followed by 40 cycles of denaturation
111 at 95°C for 3 s and annealing/extension at 60°C for 30 s. A melting curve (60–95°C) was
112 generated at the end of each run to verify specificity. A standard curve generated by 10-fold

serial dilution (from 1–10,000 ng/mL) of genomic DNA obtained from the peripheral blood leukocytes of a healthy dog was used to determine the absolute equivalent amount of cfDNA in each sample. All samples were evaluated in triplicate, and a negative control (without template) was included in each plate.

Statistical analysis

The Mann–Whitney *U* test and Kruskal–Wallis test were applied to compare plasma cfDNA concentrations between groups. The Steel–Dwass test was performed on each pair of groups following the Kruskal–Wallis test. The diagnostic values of cfDNA for the prediction of distant metastasis were assessed by using receiver operation characteristic (ROC) curves, with a minimum area under the curve (AUC) of 0.7 required for consideration of the ROC model. All analyses were performed using JMP 13 software (SAS Institute, Cary, NC), and differences were considered statistically significant at $p \leq 0.05$.

Results

Patient characteristics

The 50 patients with malignant tumors included 27 males (7 intact) and 23 females (4 intact), with a median age of 11 y (range: 1–16 y). The 13 patients with benign tumors or nodules included 7 males (3 intact) and 6 females (2 intact), with a median age of 10 y (range 4–15 y). The 11 control dogs included one intact male and 10 females (6 intact), with a median age of 6 y (range 1–12 y). The most common malignant tumor types were lymphoma or leukemia (*n* = 11), hemangiosarcoma (*n* = 8), urothelial carcinoma (*n* = 5; 4 dogs with transitional

carcinoma of the bladder and 1 dog with prostate carcinoma), mammary carcinoma (*n* = 4), oral squamous cell carcinoma (*n* = 3), apocrine gland anal sac carcinoma (*n* = 3), hepatic tumor (*n* = 3; 1 dog with hepatic cell carcinoma, 1 dog with cholangiocarcinoma, and 1 dog with carcinoid), soft tissue sarcoma (*n* = 2; 1 dog with undifferentiated sarcoma and 1 dog with nerve sheath tumor), malignant melanoma (*n* = 2), gastrointestinal stromal tumor (*n* = 2), and one each of the following: mast cell tumor, osteosarcoma, ceruminous adenocarcinoma, salivary adenocarcinoma, apocrine adenocarcinoma, pheochromocytoma, seminoma. Except for lymphoma or leukemia cases, distant metastasis at sampling was observed in 19 dogs (lung, *n* = 12; liver, *n* = 4; spleen, *n* = 2; and peritoneum, *n* = 1). The benign tumors/nodules included adenoma (*n* = 6; mammary grand, sebaceous gland, and rectal), leiomyoma (*n* = 2), and one each of the following: melanocytoma, histiocytoma, hematoma, bladder polyp, and epidermal cyst.

cfDNA concentrations and DNA integrity index

The median cfDNA concentration of LINE-99 elements in malignant, benign, and control dogs was 885 ng/mL (mean: 6,290 ng/mL; range: 51–115,000 ng/mL), 521 ng/mL (mean: 652 ng/mL; range: 166–1,870 ng/mL), and 524 ng/mL (mean: 481 ng/mL; range: 205–802 ng/mL), respectively (Fig. 1A). Plasma cfDNA concentrations were significantly elevated in dogs with malignant tumors compared with those with benign nodules ($p = 0.048$) and healthy controls ($p = 0.011$).The median DNA integrity index in malignant, benign, and control dogs was 0.62 (mean: 0.58; range: 0.14–0.95), 0.75 (mean: 0.69; range: 0.47–0.86), and 0.69

(mean: 0.72; range: 0.55–0.99), respectively (Fig. 1B). The integrity index was significantly lower in dogs with malignant tumors as compared with healthy controls ($p = 0.038$).

Among the 50 dogs with malignant tumors, lymphoma or leukemia and hemangiosarcoma resulted in significantly higher cfDNA levels compared with other malignant tumors (Fig. 2A). The integrity index for dogs with hemangiosarcoma was significantly lower compared with dogs with other malignant tumors (Fig. 2B). Among 39 dogs with malignant tumors, except for those with lymphoma or leukemia, dogs with distant metastases displayed significantly higher cfDNA levels and a lower integrity index compared with dogs without distant metastases (Fig. 3).

Diagnostic value of cfDNA for the prediction of distant metastasis

Except for lymphoma or leukemia cases, ROC curves were generated to distinguish distant metastasis patients from other patients and control dogs based on plasma cfDNA levels and the integrity index. The cut-off value for cfDNA concentration was 852 ng/mL, with an AUC of 0.833 (95% confidence interval [CI]: 0.714–0.952; $p = 0.002$), 73.7% sensitivity, and 88.6% specificity (Fig. 4A). The cut-off value of the integrity index was 0.62, with an AUC of 0.752 (95% CI: 0.611–0.893; $p < 0.001$), 63.2% sensitivity, and 81.8% specificity (Fig. 4B, Table 1).

Plasma cfDNA dynamics and tumor progression

In several patients, cfDNA concentration increased along with the progression of tumor metastasis or recurrence, whereas, the integrity index tended to decrease according to tumor

progression, with this change inversely proportional to changes in cfDNA concentration (Fig. 5). Fluctuations in cfDNA obtained from several patients were observed before a period of disease progression, and the lead time ranged from a few weeks to several months compared with clinical staging.

Discussion

We found that patients with malignant tumors had significantly higher median plasma cfDNA concentration than dogs with benign tumors or nodules or healthy controls. Several studies reported higher cfDNA concentrations in multiple human cancer patients,[26,32] and previous studies also revealed that circulating cfDNA in plasma from dogs with mammary neoplastic lesions and various neoplasias was significantly higher than in healthy dogs.[1,15] Our data showed significantly elevated levels of plasma cfDNA in dogs with lymphoma or leukemia and hemangiosarcoma compared with those harboring other malignant tumors, which agreed with a previous result concerning lymphoid neoplasia.[24] Possible explanations include the higher tumor burden associated with lymphoma or leukemia, with necrotic, apoptotic, and fragile cells frequently encountered in specimens collected from lymphoid neoplasms.[24] Additionally, canine hemangiosarcoma is a neoplasm of vascular endothelial origin that has an aggressive biological behavior and a high rate of rapid and widespread metastasis.[23] Histologically, the tumors are cellular with moderate-to-extensive areas of hemorrhage and necrosis.[13] In fact, most of the hemangiosarcoma dogs included in our study had distant metastases. Hemangiosarcoma is a systemic disease; therefore, elevated plasma cfDNA

concentration in dogs with hemangiosarcoma might be a consequence of tumor volume and tumor-cell necrosis.

We observed a significantly lower DNA integrity index in dogs with malignant tumors compared with that in healthy controls. Moreover, dogs with hemangiosarcoma had a lower DNA integrity index than dogs with other malignant tumors. In veterinary medicine, there is minimal data concerning the efficacy of the DNA integrity index, although a study of canine mammary tumors reported results similar to ours.[1] Previous studies suggest that the extent of DNA fragmentation during apoptosis might vary between cancer and normal cells, and that increased DNA fragmentation in cancer cells undergoing apoptosis relative to that in non-cancerous cells might provide an explanation for the decreased DNA integrity index in cancer patients.[7,18] In fact, some studies report either equivalent or lower DNA integrity indexes in human patients with cancer.[3,18,19] However, it remains unknown whether this theory applies to canine tumors, with a previous review suggesting that the DNA integrity index is controversial as a blood-based biomarker of human cancers.[3] Therefore, further studies are needed to evaluate the clinical utility of the DNA integrity index in veterinary medicine.

Our dogs with distant metastases had significantly higher plasma cfDNA concentrations and a lower DNA integrity index relative to patients with malignant disease but no distant metastases. Both markers showed good predictive capability, with the AUC for the cfDNA concentration generated by the LINE-99 primers higher than that for the DNA integrity index. Based on the cut-off value of 852 ng/mL for plasma cfDNA, we suggest that

patients with distant metastasis might be discriminated from other dogs with 73.7% sensitivity and 88.6% specificity when using LINE-99 primers for qPCR analysis. Previous studies suggest that plasma cfDNA levels correlate with larger tumor size, metastasis, and later cancer stage.[6,17] Additionally, studies reported that elevated cfDNA levels were significantly correlated with poor outcomes, and that cfDNA quantification could be used as an effective biomarker to discriminate patients with several malignancies from healthy individuals.[18,27,32] Our results from canine patients indicated cfDNA as a good screening tool for the detection of distant metastasis and possible utility when used in combination with existing diagnostic tools, including thoracic radiography and abdominal ultrasound, to improve diagnostic efficacy.

By tracking the plasma cfDNA concentration and DNA integrity index at several times in 6 patients, we found that cfDNA parameters correlated well with the clinical stage and tended to increase during or before periods of disease progression, suggesting comparability in monitoring the clinical stage. In contrast, we found that cfDNA parameters did not reflect the disease progression observed in patient 5. Patient 4 did not appear to develop disease progression during the study period. A previous study reported that cfDNA could be used to monitor disease progression in non-small-cell lung cancer patients;[32] however, several other studies demonstrated that circulating tumor DNA using mutated genes or the Epstein-Barr virus gene was superior to nonspecific cfDNA as an early marker of relapsed and refractory disease.[8,12] To our knowledge, evaluation of the efficacy of cfDNA

monitoring as a disease-response biomarker associated with several canine malignancies has not been reported previously. However, although the integrity index tended to decrease according to tumor progression in a few of our patients, mismatched changes with tumor progression were observed in other patients.

Our study had several limitations. First, the small sample size used for each analysis might have limited the extent to which differences between groups could be detected. Additionally, our study included various tumor types; therefore, our findings should be verified in a larger population and unified according to tumor type. Also, our study was a retrospective analysis, and imaging modalities for each cancer stage were not standardized. This might have resulted in underestimation of distant metastasis in some patients; therefore, a future prospective study involving standardized imaging modalities is necessary.

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331

Table 1. AUC of cfDNA and the DNA integrity index for screening distant metastasis.

	cfDNA	DNA integrity index
AUC (95% CI)	0.833 (0.714–0.952)	0.752 (0.611–0.893)
Cut off value	852 ng/mL	0.62
Accuracy (%)	84.1	71.4
Sensitivity (%)	73.7	63.2
Specificity (%)	88.6	81.8
PPV (%)	73.7	52.0
NPV (%)	88.6	84.2

AUC = area under the receiver operating characteristics curve; cfDNA = cell-free DNA; CI = confidence interval; NPV = negative predictive value; PPV = positive predictive value.

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Table 2. Clinical characteristics of 6 patients assessed during follow-up study.

Patient	Age (y)	Sex/ status	Breed	Tumor site	Tumor type	Stage[a)]	1st-line treatment	2nd-line treatment	Metastasis site, timing	Recurrence, timing
1	10	Castrated	Mix	Urinary bladder	TCC	T2N0M0	Surgery	COX2i	Lung, day102	-
2	10	Castrated	FB	Subcutaneous	STS	T4N0M0	Surgery	LDCPA	-	Local site, day48
3	13	Spayed	Mix	Spleen	HS	T2N0M0, stage II	Surgery	DOX	Peritoneum, day60	-
4	8	Male	Mix	MC	LSA	Stage Vb	Chemotherapy	-	-	-
5	4	Castrated	FB	MC	LSA	Stage Va	Chemotherapy	-	-	Mandibular LN, day220
6	8	Castrated	CHH	Spleen, liver, PB, LN	Leukemia	-	Chemotherapy	-	-	Spleen, Liver, PB, day205

a) According to the WHO staging system.[23]

CHH = Chihuahua; COX2i = cyclooxygenase-2 inhibitor; DOX = doxorubicin; FB = French Bulldog; HS = hemangiosarcoma; LDCPA = low-dose cyclophosphamide; LN = lymph node; LSA = lymphoma; MC = multicentric; PB = peripheral blood; STS = soft tissue sarcoma; TCC = transitional cell carcinoma.

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Figure 1. A. Box-plot of plasma cfDNA concentrations in dogs with malignant tumors or benign diseases and healthy controls. **B.** cfDNA-integrity index. Each box indicates the 25th and 75th percentiles. The horizontal line inside the box indicates the median, and the whiskers indicate the extreme measured values.

cfDNA = cell-free DNA.

Figure 2. A. Box-plot of plasma cfDNA concentrationsin dogs with LSA, HS, and other malignant tumors. **B.** cfDNA-integrity index. Each box indicates the 25th and 75th percentiles. The horizontal line inside the box indicates the median, and the whiskers indicate the extreme measured values.

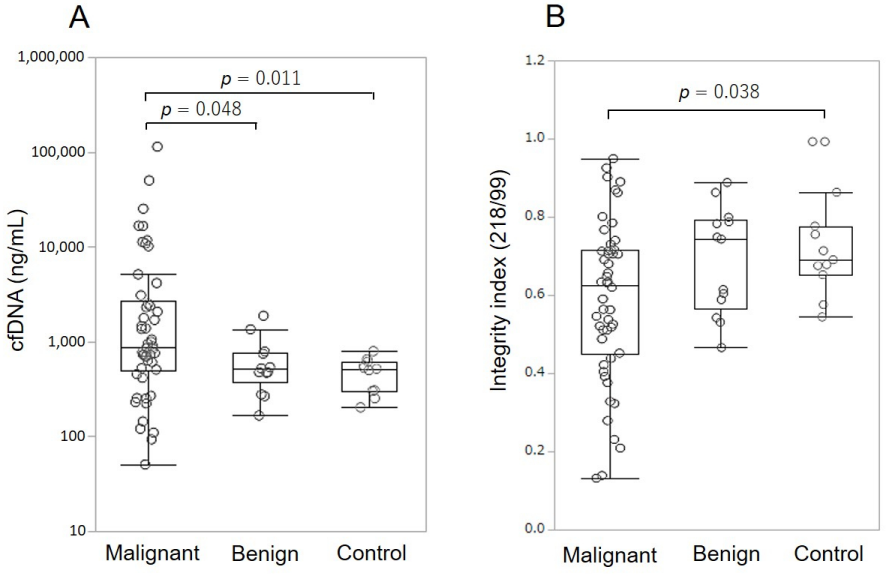
cfDNA, cell-free DNA; HS, hemangiosarcoma; LSA, lymphoma/leukemia.

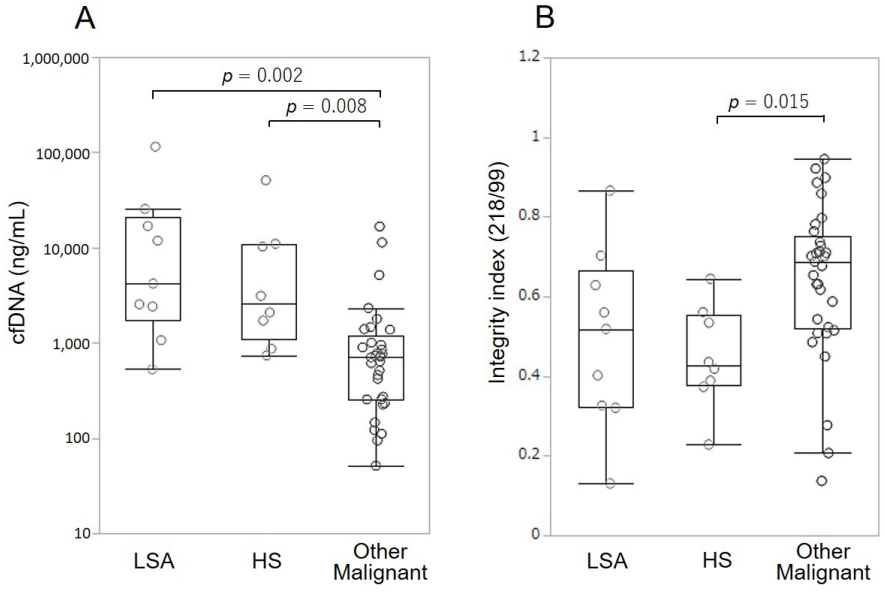
Figure 3. A. Box-plot of plasma cfDNA concentrations in malignant-tumor dogs with DM or without DM. **B.** cfDNA-integrity index. Each box indicates the 25th and 75th percentiles. The horizontal line inside the box indicates the median, and the whiskers indicate the extreme measured values. cfDNA, cell-free DNA; DM, distant metastasis.

Figure 4. Receiver operating characteristic and AUC of plasma cfDNA concentrations (**A**) and cfDNA-integrity index (**B**) to distinguish patients with distant metastasis from other patients and controls. The AUC for cfDNA concentrations and the cfDNA-integrity index was 0.833 (95% CI: 0.714–0.952; $p = 0.002$) and 0.752 (95% CI: 0.611–0.893; $p < 0.001$), respectively.

AUC, area under the curve; cfDNA, cell-free DNA; CI, confidence interval.

Figure 5. Comparison of cfDNA and cfDNA-integrity index according to treatment response. cfDNA levels (solid line) and the cfDNA-integrity index (dotted line) at various time points in 6 patients. The vertical axis on the left represents cfDNA concentration, and the vertical axis on the right represents the cfDNA-integrity index. The horizontal axis labels represent the time points of plasma-sampling day and tumor status. **A.** Patient 1, transitional carcinoma of the bladder treated with surgery and cyclooxygenase-2 inhibitor; **B.** Patient 2, undifferentiated sarcoma treated with surgery; **C.** Patient 3, splenic hemangiosarcoma treated with surgery and chemotherapy; **D.** Patient 4, lymphoma treated with chemotherapy; **E.** Patient 5, lymphoma treated with chemotherapy; **F.** Patient 6, leukemia treated with chemotherapy. CR, complete response; PD, progressive disease; PR, partial response.


A
1,000,000
100,000
10,000
1,000
100
10
cfDNA (ng/mL)
p = 0.011
p = 0.048
Malignant
Benign
Control
B
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Integrity index (218/99)
p = 0.038
Malignant
Benign
Control


A
1,000,000
100,000
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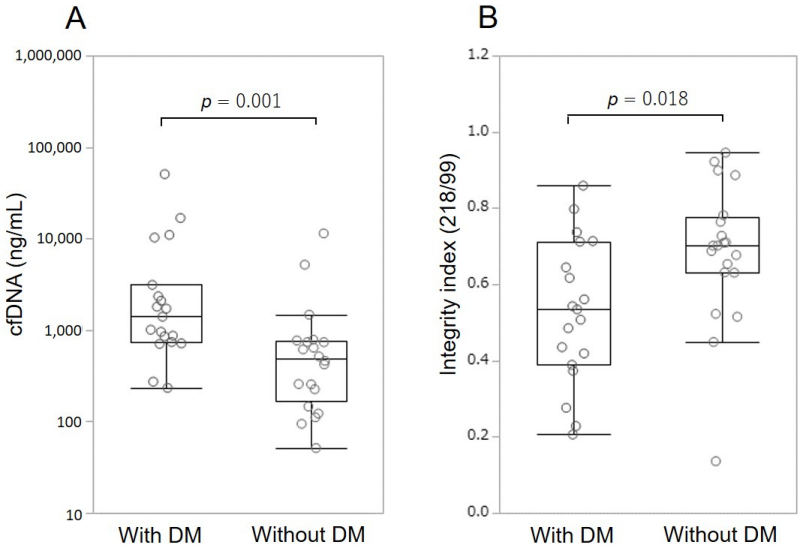
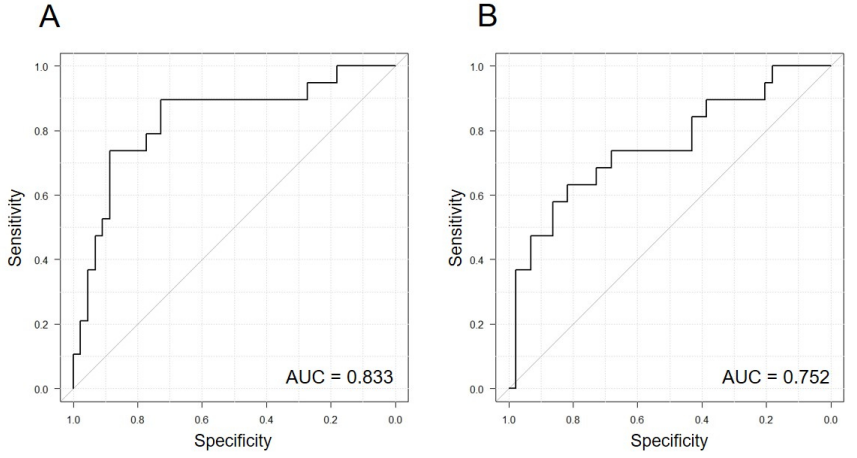
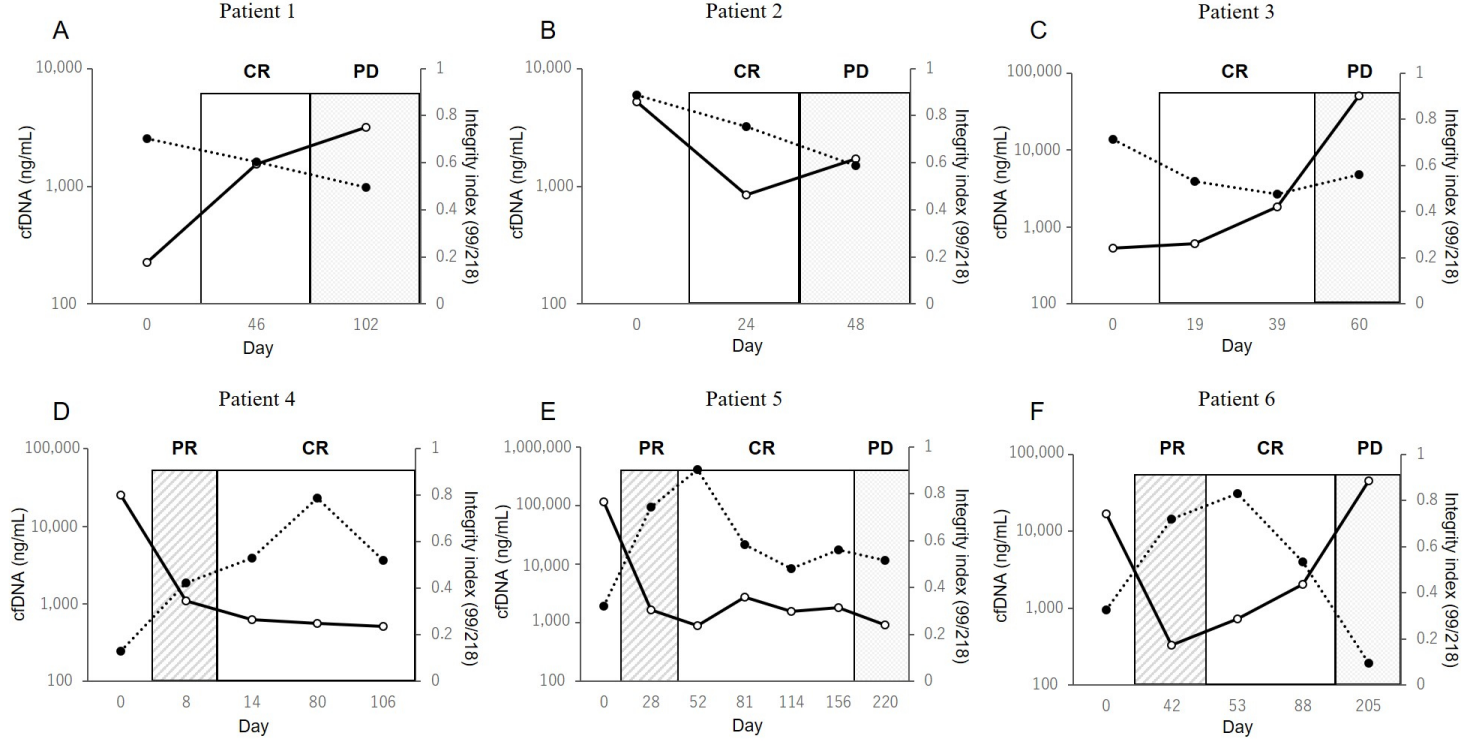
1,000
100
10
cfDNA (ng/mL)
p = 0.002
p = 0.008
LSA
HS
Other Malignant
B
1.2
1
0.8
0.6
0.4
0.2
0
Integrity index (218/99)
p = 0.015
LSA
HS
Other Malignant


A
1,000,000
100,000
10,000
1,000
100
10
cfDNA (ng/mL)
p = 0.001
With DM
Without DM
B
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Integrity index (218/99)
p = 0.018
With DM
Without DM


A
B
Sensitivity
Specificity
1.0
0.8
0.6
0.4
0.2
0.0
AUC = 0.833
AUC = 0.752


A
Patient 1
CR
PD
10,000
1,000
100
cfDNA (ng/mL)
1
0.8
0.6
0.4
0.2
0
Integrity index (99/218)
0
46
102
Day
B
Patient 2
CR
PD
10,000
1,000
100
cfDNA (ng/mL)
1
0.8
0.6
0.4
0.2
0
Integrity index (99/218)
0
24
48
Day
C
Patient 3
CR
PD
100,000
10,000
1,000
100
cfDNA (ng/mL)
1
0.8
0.6
0.4
0.2
0
Integrity index (99/218)
0
19
39
60
Day
D
Patient 4
PR
CR
100,000
10,000
1,000
100
cfDNA (ng/mL)
1
0.8
0.6
0.4
0.2
0
Integrity index (99/218)
0
8
14
80
106
Day
E
Patient 5
PR
CR
PD
1,000,000
100,000
10,000
1,000
100
cfDNA (ng/mL)
1
0.8
0.6
0.4
0.2
0
Integrity index (99/218)
0
28
52
81
114
156
220
Day
F
Patient 6
PR
CR
PD
100,000
10,000
1,000
100
cfDNA (ng/mL)
1
0.8
0.6
0.4
0.2
0
Integrity index (99/218)
0
42
53
88
205
Day