



■ ONCOLOGY

Detection and utility of cell-free and circulating tumour DNA in bone and soft-tissue sarcomas

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Aims

Cell-free DNA (cfDNA) and circulating tumour DNA (ctDNA) are used for prognostication and monitoring in patients with carcinomas, but their utility is unclear in sarcomas. The objectives of this pilot study were to explore the prognostic significance of cfDNA and investigate whether tumour-specific alterations can be detected in the circulation of sarcoma patients.

Methods

Matched tumour and blood were collected from 64 sarcoma patients (n = 70 samples) prior to resection of the primary tumour (n = 57) or disease recurrence (n = 7). DNA was isolated from plasma, quantified, and analyzed for cfDNA. A subset of cases (n = 6) underwent whole exome sequencing to identify tumour-specific alterations used to detect ctDNA using digital droplet polymerase chain reaction (ddPCR).

Results

Cell-free was present in 69 of 70 samples above 0.5 ng/ml. Improved disease-free survival was found for patients with lower cfDNA levels (90% vs 48% at one-year for ≤ 6 ng/ml and > 6 ng/ml, respectively; $p = 0.005$). Digital droplet PCR was performed as a pilot study and mutant alleles were detectable at 0.5% to 2.5% of the wild type genome, and at a level of 0.25 ng tumour DNA. Tumour-specific alterations (ctDNA) were found in five of six cases.

Conclusion

This work demonstrates the feasibility and potential utility of cfDNA and ctDNA as biomarkers for bone and soft-tissue sarcomas, despite the lack of recurrent genomic alterations. A larger study is required to validate these findings.

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Keywords: Sarcoma, Cell-free DNA, Circulating tumour DNA

Article focus

- Does the amount of cell-free DNA (cfDNA) in circulation at diagnosis have prognostic implications in sarcoma?
- Can tumour-specific alterations be detected in the circulation of sarcoma patients?

Key messages

- Low levels of cfDNA in a patient's plasma at diagnosis may correlate with improved disease-free survival in sarcoma.
- Circulating tumour DNA is detectable even in sarcomas without known recurrent genomic alterations.

Strengths and limitations

- All results were obtained using human samples.
- This is a pilot study and results should be verified in a larger patient cohort.

Introduction

Fragmented DNA is released into the bloodstream through cellular processes such as apoptosis, necrosis, and secretion. This cell-free DNA (cfDNA) is mainly derived from cells of the haematopoietic system and mean levels in healthy individuals range between 3 ng and 7 ng per ml plasma.^{1,2} Several factors influence these levels including exercise³ and infection,⁴ which have been shown to elevate cfDNA concentrations. Raised cfDNA levels

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are also observed in patients with cancer, who may also have cfDNA derived from tumour cells in their circulation.⁵⁻⁷ This circulating tumour DNA (ctDNA) carries the mutational signature of the patient's tumour.^{8,9} As such, ctDNA has potential as a tumour-specific biomarker with diagnostic utility in both disease detection and monitoring. Despite its low abundance, detection of ctDNA has been made possible given advances in techniques such as next-generation sequencing (NGS) and digital droplet polymerase chain reaction (ddPCR). NGS enables identification of tumour-specific alterations and with high sensitivity rare mutation detection by ddPCR, these tumour-specific alterations allow ctDNA molecules to be distinguished among a wild type background.^{10,11}

Both cfDNA and ctDNA are active areas of research in many types of cancer due to their value as noninvasive diagnostic tools, termed liquid biopsies. They have been shown to correlate with disease burden, have prognostic implications in different carcinomas, and are being explored for detection of minimal residual disease post-resection of the primary tumour.¹²⁻¹⁵ However, evaluation of these analytes in sarcoma has been underexplored. Sarcomas are a rare, diverse group of cancers of mesenchymal origin with more than 70 distinct subtypes that account for approximately 1% of all adult solid tumours. Despite adequate management of the primary tumour, typically with wide surgical resection plus perioperative radiation and/or chemotherapy, there remains a high incidence of tumour relapse. For soft-tissue sarcomas (STSs), the rates of local recurrence and metastatic disease are estimated at 10% to 30% and 35% to 40%, respectively.¹⁶ Investigations into liquid biopsies for sarcoma have mainly focused on subtypes with recurrent genomic alterations, such as chondrosarcoma (isocitrate dehydrogenase type 1),¹⁷ myxoid liposarcoma (t(12;16)),¹⁸ and synovial sarcoma (t(X;18)).¹⁸ However, the majority of adult STS subtypes, such as undifferentiated pleomorphic sarcoma and myxofibrosarcoma, lack any recurrent alterations,¹⁹ and for these entities no analytically valid approaches have been presented.

While biopsy of the primary tumour will likely remain the gold standard for diagnosis in sarcoma, cfDNA/ctDNA testing has advantages which encourage continued study as an adjunct prognostic and monitoring tool. These include its short half-life (30 to 120 mins),²⁰ and the minimally invasive nature of a blood draw compared with tissue biopsy. Serial cfDNA/ctDNA analyses provide a means of real-time monitoring of treatment response and early detection of recurrence. This approach has been explored in several common cancers,^{21,22} including a case study of a patient with high-grade malignant spindle cell sarcoma.²³ For cfDNA/ctDNA monitoring to be incorporated into routine sarcoma care, extensive evaluation of this biomarker will be required across many sarcoma subtypes of varied grade and progression, with a focus on developing an approach for those without recurrent genomic alterations.

The objectives of this exploratory study were two-fold, to: 1) explore the prognostic significance of cfDNA in bone and STSs; and 2) investigate whether tumour-specific alterations can be detected in the plasma of sarcoma patients using ddPCR.

Methods

Patient identification, sample collection, and processing. As part of a larger institutional tissue and blood biobanking programme, we carried out a prospective cohort study of adult patients that presented with a suspected new or recurrent limb bone or STS. Patients with retroperitoneal tumours were excluded. All patients gave written informed consent under approved institutional ethics board protocols (REB# 01-0138 U) and studies were conducted according to the Declaration of Helsinki.²⁴ All identified subjects had 20 ml of whole blood collected into EDTA tubes in the operating room, prior to either open biopsy or definitive surgical resection, as tumour manipulation may increase cfDNA/ctDNA levels.²⁵ Blood samples were processed within one hour of collection. Plasma was separated by centrifugation at 2,500 g for ten minutes, transferred to microfuge tubes, and centrifuged at 16,000 g for ten minutes to remove cellular debris. The resultant plasma samples were stored in 1 ml aliquots at -80°C until DNA extraction. Tumour tissue and peripheral blood were also collected and banked during this procedure. In addition, baseline demographic details (patient age and sex) and oncological variables (tumour size, location, histology, stage) were collected. Following surgical management, patients were followed prospectively according to our institutional protocol with physical examinations and chest radiographs for those with STS or chest CT scans for those with bone sarcomas every three months until year 2, every six months from year 2 to 5, and annually from year 5 to 10 postoperatively.

While all patients with suspected sarcoma (based on clinical assessment and imaging findings) who provided informed consent had blood and tumour samples banked following their biopsy or resection procedures, inclusion in the analysis required diagnosis of a bone or STS by pathological review of the biopsy or surgically resected specimen. Excluded were three samples from patients ultimately diagnosed with carcinoma (n = 2) and lymphoma (n = 1).

Isolation and quantification of cell-free plasma DNA. Cell-free DNA was isolated from 2 ml to 3 ml of plasma using QIAamp circulating nucleic acid extraction kits (Qiagen, USA) with the use of the QIAvac system. Extractions were performed according to the manufacturer's instructions with the following modifications: no carrier RNA was added; DNA columns were eluted with 60 µl of buffer RNAase-free water with 0.04% sodium azide; eluate was reapplied to the column following first elution, incubated, and eluted a second time by centrifugation. Extracts were stored at -80°C until use. The concentration of purified

plasma DNA was determined by quantitative PCR (qPCR) using an 81 bp amplicon of the *EIF2C1* gene on chromosome 1 and a dilution series of placenta DNA.²⁶ Duplicate qPCR assays were performed for each of the five-point standard curve and test samples. PowerSyber Green master mix (Applied Biosystems, USA) was used for all PCR reactions, and assays were performed on CFX96 Real Time PCR Detection System (Bio-Rad, USA).

Quality assessment of total DNA in samples. Capillary electrophoresis analysis of 60 samples was performed at the Princess Margaret Genomics Centre using the High Sensitivity DNA chip and 2100 Bioanalyzer (Agilent Technologies, USA). Sample concentrations of 1 ng/ul were achieved by speed vacuuming and dilution with distilled water. Quality assessment was not performed in cases due to insufficient amounts of DNA.

Identification of tumour-specific DNA variants. DNA was isolated from freshly frozen tumour samples and matched blood using the DNeasy Kit (Qiagen). Whole exome sequencing (WES) was performed on six matched tumour-blood DNA samples to identify tumour-specific genetic alterations including single nucleotide variants (SNVs) using the Illumina platform (USA). SNVs identified from WES analysis were used to design primers and probes to detect the tumour-specific alterations in the corresponding cfDNA in the six cases using ddPCR.

Digital droplet PCR assay design. Primer sets and allele specific probes were designed and synthesized by Integrated DNA Technologies (IDT) Canada for each of the paired patient samples based on WES analysis (Supplementary Table i). The ddPCR reactions were performed using the QX200 AutoDG Droplet Digital PCR System (Bio-Rad) and conducted using a temperature gradient of 60°C to 50°C. Hexachloro-fluorescein (HEX) and 6-carboxyfluorescein (FAM) intercalating fluorophores were employed for wild type and mutant alleles, respectively. For five cases (#1 to #5), cfDNA was isolated from plasma while for case #6, cfDNA was isolated from serum.

Statistical analysis. The statistical analysis was performed using Prism 8 (GraphPad Software, USA). Survival was assessed as the time between the date of surgical resection and the date of death of any cause (overall survival) or date of metastasis or local recurrence (disease-free survival). Outcomes for living patients were censored at the time of their last follow-up examination. Analysis of clinical variables associated with survival was performed using the Kaplan-Meier method (log-rank test). The presence of differences between groups was evaluated using an unpaired *t*-test, chi-squared test, or Fisher's exact test, as appropriate. Statistical significance was set at $p < 0.05$.

Results

Cell-free DNA detection in plasma samples. In total, 70 plasma samples from 64 patients with limb sarcomas were included in this pilot study. Demographic and oncological variables for the patient cohort are included in Table I. There were 54 cases of STS and ten of bone

Table I. Demographic and oncological variables for all patients included in the study. A total of 70 plasma samples were analyzed from 64 patients.

Variable	Result
Mean age, yrs (range)	63 (18 to 90)
Sex, n (%)	
Male	37 (58)
Female	27 (42)
Histology, n (%)	
STS	
UPS	12 (19)
MFS	15 (23)
LPS	9 (14)
MLS	3 (5)
LMS	3 (5)
Other	12 (19)
Bone sarcoma	
OSA	5 (8)
CSA	4 (6)
Other	1 (2)
Grade, n (%)	
1	8 (13)
2	20 (31)
3	35 (55)
Presenting status, n (%)	
M0	50 (78)
M1	7 (11)
LR ± Mets	7 (11)
Location, n (%)	
Deep	56 (88)
Superficial	8 (13)
Maximum diameter, cm (range)	12.7 (2.0 to 34.4)
Patient treatment received prior to sample collection, n (%)	
None	47 (73)
Radiation	13 (20)
Chemotherapy	3 (5)
Both	1 (2)
Surgical procedure performed at time of sample collection, n (%)	
Open biopsy	18 (28)
Resection	45 (70)

cfDNA, cell-free DNA; CSA, chondrosarcoma; LMS, leiomyosarcoma; LPS, liposarcoma; LR ± Mets, presented with a local recurrence and/or metastases from a previously treated sarcoma; M0, no metastases at presentation; M1, metastases at presentation; MFS, myxofibrosarcoma; MLS, myxoid liposarcoma; OSA, osteosarcoma; STS, soft-tissue sarcoma; UPS, undifferentiated pleomorphic sarcoma.

sarcoma. The majority (35/64; 55%) were Grade 3, localized at presentation (50/64; 78%), and did not receive chemotherapy or radiation prior to sample collection (47/64; 73%). Mean follow-up after definitive resection of the primary sarcoma was 13.6 months (0 to 129).

Plasma samples were analyzed by qPCR to confirm the presence of cfDNA and to assess the concentration of DNA in each sample. Of the samples tested, all but one (a case of Grade 1 chondrosarcoma) were positive for cfDNA at levels above 0.5 ng per ml. The majority of values were clustered between 2 and 14 ng per ml (54 of 70 samples;

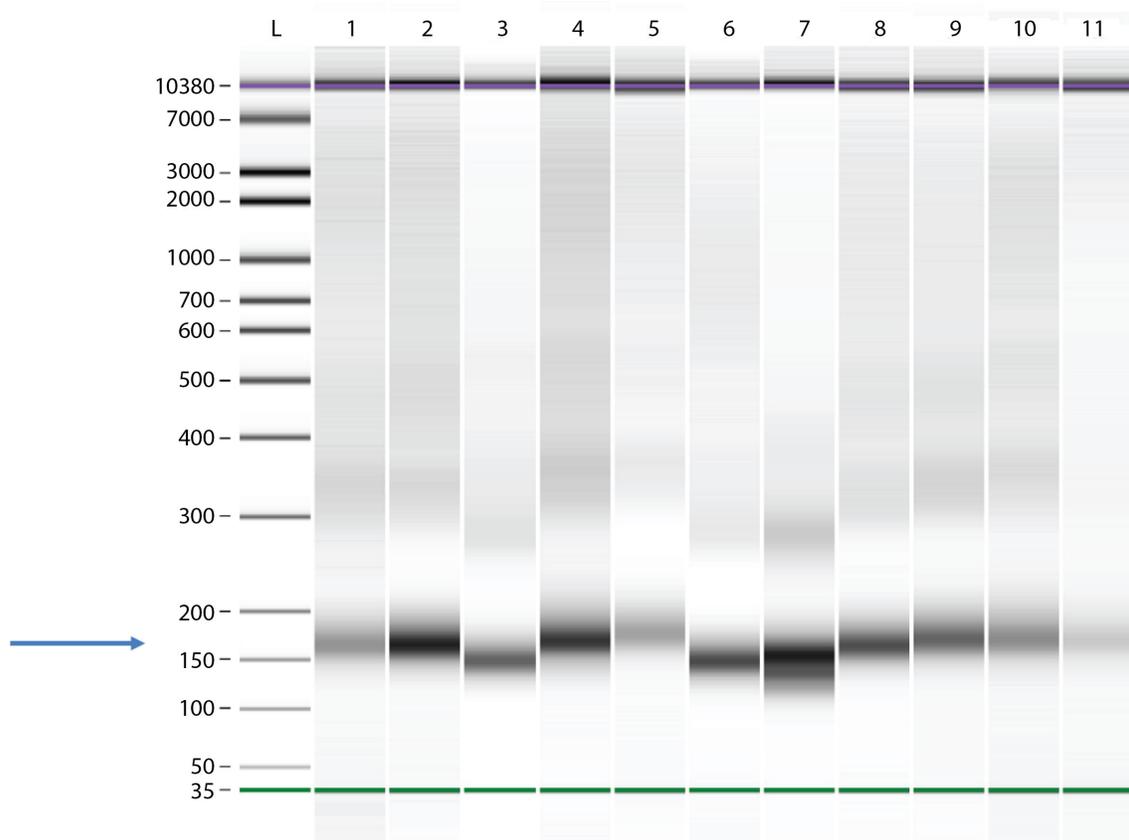


Fig. 1

Cell-free DNA fragment size assessment by capillary electrophoresis. Lane L contains the molecular weight ladder; Lanes 1 to 11 contain extracted DNA samples for 11 individual cases. All samples tested contained signal peaks corresponding to DNA fragments of 140 bp to 200 bp, consistent with cell-free DNA (blue arrow). The purple lines indicate the upper marker (10,380 bp) while the green lines indicate the lower marker (35 bp).

77%) and the median was 6 ng/ml (interquartile range (IQR) 3.44 to 11.35). Six patients had cfDNA isolated at two separate timepoints. For four patients, samples were collected prior to both the open biopsy and the definitive resection. One of these patients received radiation following the biopsy and prior to the resection, while the other three did not have any neoadjuvant treatment. A fifth patient had cfDNA isolated at resection of the primary tumour and 16 months later when the patient developed a local recurrence. The final patient had multiple metachronous metastases from dedifferentiated liposarcoma and had plasma collected prior to two of the metastasectomies. For all six patients, cfDNA was detectable at both timepoints at levels above 2 ng/ml and in all cases, the first result was used in subsequent analyses (see Supplementary Table ii).

Cell-free DNA quality assessment. High-resolution separation and quantitation of DNA down to pg/ μ l sensitivity was accomplished by capillary electrophoresis (CE) as shown in Figure 1. All 60 samples tested showed large signal peaks corresponding to small DNA fragments of approximately 140 bp to 200 bp in size, characteristic of cfDNA.²⁷ For the majority of samples, the small DNA fragments generating these peaks were present in concentrations greater than all other fragment sizes detected.

Association of cfDNA with patient survival. Overall and disease-free survival for the entire cohort was 86% and 71% at one year post-definitive resection, respectively. Improved overall survival was associated with younger age ($p = 0.002$), female sex ($p = 0.041$), localized disease at presentation ($p < 0.001$), and smaller tumour size ($p = 0.025$) in univariate analysis. No patient or oncological baseline characteristics correlated with disease-free survival. In order to investigate the association between cfDNA at presentation and oncological outcome, patients were divided into two groups using the cohort median of 6 ng/ml (IQR 3.44 to 11.35) as the cut-off. Disease-free survival was significantly greater for patients that had ≤ 6 ng/ml cfDNA compared to those with > 6 ng/ml ($p = 0.005$, univariate analysis) as shown in Figure 2a and Table II; however, a statistically significant difference in overall survival was not observed (Figure 2b, Table II; $p = 0.064$, univariate analysis).

Presenting status was the only baseline characteristic for which there was a difference between patients with > 6 ng/ml cfDNA at presentation versus those with ≤ 6 ng/ml cfDNA. There were more patients that presented with metastatic disease in the higher cfDNA group than in the lower cfDNA group (22% vs 0%, respectively; $p = 0.026$, chi-squared test). No differences in histology, grade, location, or treatment received prior to sample processing

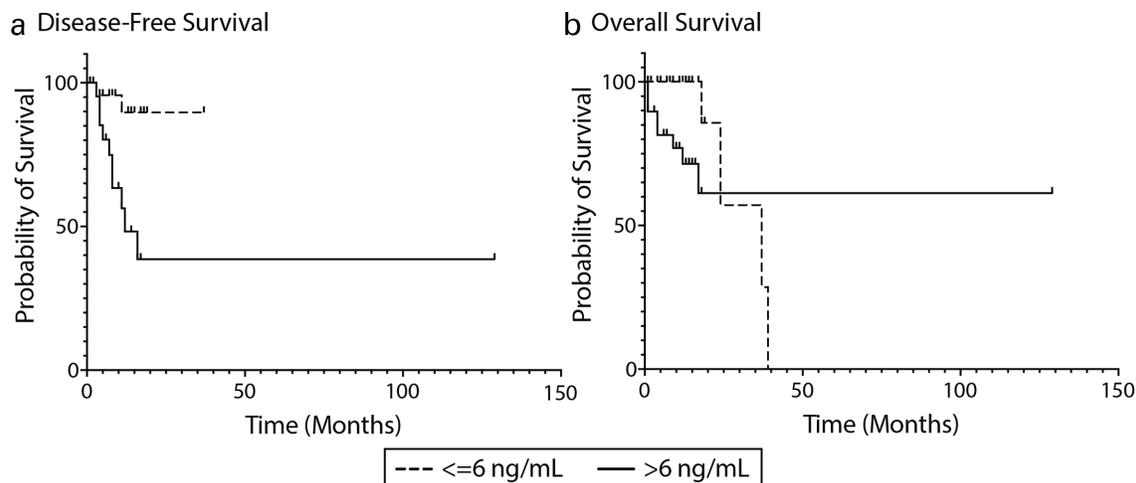


Fig. 2

Disease-free and overall survival for sarcoma patients depending on amount of cell-free DNA (cfDNA) at time of surgery. a) Patients with ≤ 6 ng/ml cfDNA have significantly better disease-free survival than patients with > 6 ng/ml cfDNA (90% vs 48% at one year for ≤ 6 ng/ml and > 6 ng/ml, respectively; $p = 0.005$, univariate analysis). b) With available numbers, a significant difference in overall survival was not detected in patients based on the level of cfDNA ($p = 0.064$, univariate analysis).

Table II. Survival analysis for the patient cohort.

Variable	N (%)	One-year DFS (%)	p-value*	One-year OS (%)	p-value*
Age (yrs)			0.962		0.002
≤ 66	33 (52)	69		100	
> 66	31 (48)	71		71	
Sex			0.508		0.041
Male	37 (58)	68		80	
Female	27 (42)	73		95	
Grade			0.055		0.138
1	8 (13)	100		100	
2	20 (31)	77		91	
3	35 (55)	52		79	
Presenting status					< 0.001
M0	50 (78)	N/A		95	
M1/LR/Metastatic	14 (22)	N/A		51	
Tumour size (cm)			0.086		0.025
≤ 11.4	31 (48)	79		96	
> 11.4	30 (47)	62		75	
cfDNA (ng/ml)			0.005		0.064
≤ 6	32 (50)	90		100	
> 6	32 (50)	48		71	

*Univariate analysis.

cfDNA, cell-free DNA; DFS, disease-free survival; LR, local recurrence; M0, no metastases at presentation; M1, metastases at presentation; N/A, not applicable; OS, overall survival.

were found (Supplementary Table iii). Mean cfDNA levels in patients who received radiation prior to sample collection was 9.8 ng/ml versus 8.5 ng/ml for those who did not ($p = 0.705$, unpaired, two-tailed t -test). Median follow-up was 13.0 months (IQR 4.8 to 17.0) for the entire cohort and did not differ between the groups ($p = 0.971$). **Circulating tumour DNA detection.** A set of pilot experiments were conducted to determine whether tumour-specific genetic alterations could be detected in the circulation of patients newly diagnosed with sarcomas that do not have known recurrent alterations. We had previously

conducted whole exome sequencing on matched tumour and blood samples and selected six cases for which cfDNA had been isolated for inclusion in this study. All cases were of high-grade sarcoma but varied with regards to presenting status and subtype (Table III). Genomic comparison of matched tumour and normal blood identified tumour-specific genetic alterations to be used as unique biomarkers for ctDNA detection. Tumour-specific targets were different for each case and were selected based on read depth of sequencing and to cover a range of variant allele frequencies (24% to 86%). Six sequence variants

Table III. Tumour-specific variants detected by whole exome sequencing of six primary tumours were used to design specific primers/probes for digital droplet polymerase chain reaction to detect circulating tumour DNA.

Case	Sarcoma subtype	Grade	Presenting status	Gene	Single nucleotide variant	Variant allele frequency
1	MFS	3	M1	SMAD4	T1584C	86%
2	UPS	3	M0	COL19A1	G1252A	50%
3	MLS	2	M0	ADGRG4	T6361C	65%
4	MFS	2	M0	HECW1	C1874A	25%
5	MFS	3	Metastatic	FOXR2	C130G	24%
6	OSA	3	Metastatic	DDX3X	A1038C	54%

Metastatic refers to distant recurrence.

M0, primary tumour, localized; M1, primary tumour, metastatic; MFS, myxofibrosarcoma; MLS, myxoid liposarcoma; OSA, osteosarcoma; UPS, undifferentiated pleomorphic sarcoma.

on SMAD4, COL19A1, ADGRG4, HECW1, FOXR2, and DDX3X genes (Table III) were ultimately used to design primers and probes for ddPCR (Supplementary Table I).

We first investigated the sensitivity of this assay by generating a series of tumour DNA mixtures containing different fractions of blood and tumour DNA (ranging from 100% to 0.5% tumour DNA) and performing ddPCR on each combination. Altered alleles were detected at 0.5% of the wild type genome for the tumour DNAs that had 50% to 86% variant allele frequency and 2.5% for the tumour DNAs that had 24% to 25% variant allele frequency of the corresponding alterations. Results for the COL19A1- G1252A alteration are shown as a representative case in Figure 3a. Similarly, we investigated the minimum input DNA required for detection of the altered allele with ddPCR by performing a set of serial dilution assays. Altered alleles were detectable for all variants at a level of 0.25 ng of tumour DNA (Supplementary Figure a) and the fractional abundance of the altered alleles remained relatively constant even with low amounts of DNA (data not shown).

We then analyzed the cfDNA isolated from each patient's blood. The assays were performed using 10 ng of cfDNA and repeated using smaller amounts (1.25 ng to 5 ng) if sufficient material remained. We were able to detect tumour-specific variants in five of the six cases, regardless of the amount of input DNA used (ranging from 1.25 ng to 10 ng; Figure 3b).

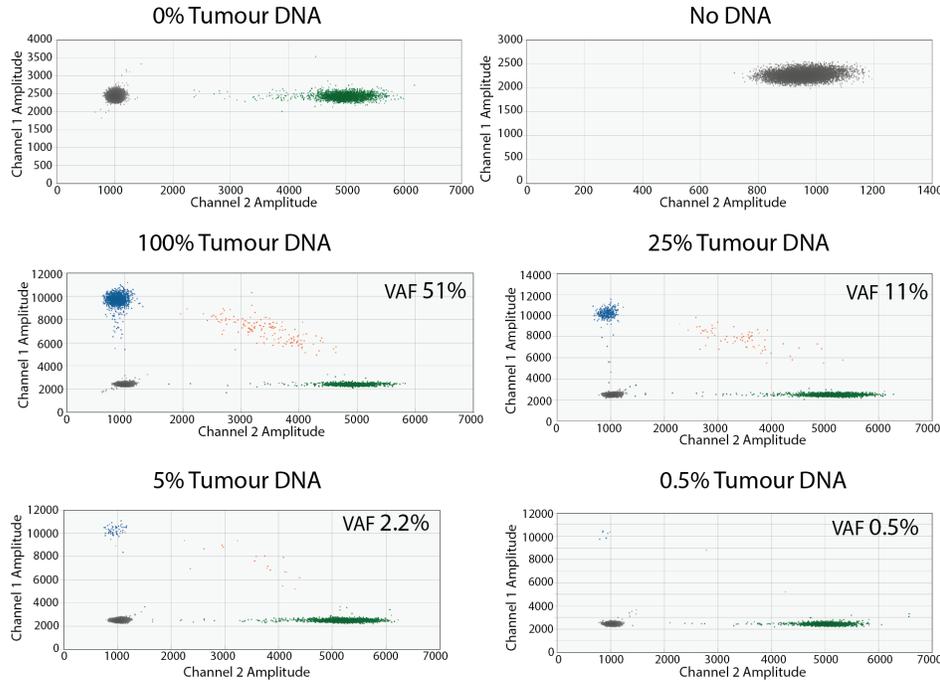
Discussion

Bone and soft-tissue sarcomas are aggressive cancers associated with high rates of local and distant failure even following initial complete resection of a localized primary tumour. New methods are required to prognosticate and surveil these patients. In cancer patients, plasma cfDNA contains both the normal short fragment, double-stranded DNA released by normal cells as well as tumour-derived DNA. Plasma cfDNA has been shown to reflect tumour burden and be prognostic in carcinomas,¹²⁻¹⁵ but has not been thoroughly investigated in sarcoma. In this pilot study, we isolated and analyzed cfDNA from 70 plasma samples taken from 64 sarcoma patients prior to resection of their tumours. DNA quantitation by qPCR

indicated positive values of cfDNA in 69 of 70 samples while the median concentration was 6 ng/ml. In the majority of cases, the dominant peak was in the range of 140 bp to 200 bp, characteristic of cfDNA.^{27,28}

We found an association between higher cfDNA and worse disease-free survival in sarcomas. Those with greater than the median amount of cfDNA at diagnosis had worse disease-free survival compared to those with less than the median ($p = 0.005$). A similar trend was observed for overall survival, however, that association was not significant ($p = 0.06$). A cutoff of 6 ng/ml to differentiate patients with 'high' versus 'low' amounts of cfDNA is reasonable based on the literature as reported values in healthy controls vary between 3 and 7 ng/ml,^{1,2} but may be revised following assessment of a larger cohort with longer follow-up. No differences in patient or oncological baseline characteristics were found between patients with ≤ 6 ng/ml cfDNA except for presenting status, as all patients with metastases at diagnosis had greater than 6 ng/ml cfDNA in their plasma. We acknowledge that this pilot study may be underpowered to detect these differences. For instance, while tumour response to neoadjuvant radiotherapy corresponds to lower cfDNA and ctDNA levels in other malignancies,^{29,30} an association was not detected in this work ($p = 0.705$). To our knowledge, only two prior studies have examined cfDNA in patients with sarcoma. Eastley et al¹ reported on total cfDNA levels in 11 patients who presented with metastatic STS and found a weak correlation with disease burden based on RECIST 1.1 criteria.³¹ In another study, Braig et al¹⁸ compared cfDNA in STS patients with active disease ($n = 64$) versus those who were in remission ($n = 19$). A third group consisted of healthy controls ($n = 41$). The authors found significantly higher amounts of cfDNA in patients with active disease (274 ng/ml) than in those who were in remission (65 ng/ml) or unaffected (67 ng/ml). It is unclear why cfDNA values in that study were so much higher than those in our work and that of others. None of these studies examined disease-free or overall survival in relation to levels of cfDNA, although this association has been documented for pancreatic cancer, non-small cell lung cancer, colorectal cancer, melanoma, and glioblastoma multiforme.¹²⁻¹⁵

a Assay sensitivity (COL19A1-G125A variant)



b COL19A1-G125A variant detection in ctDNA

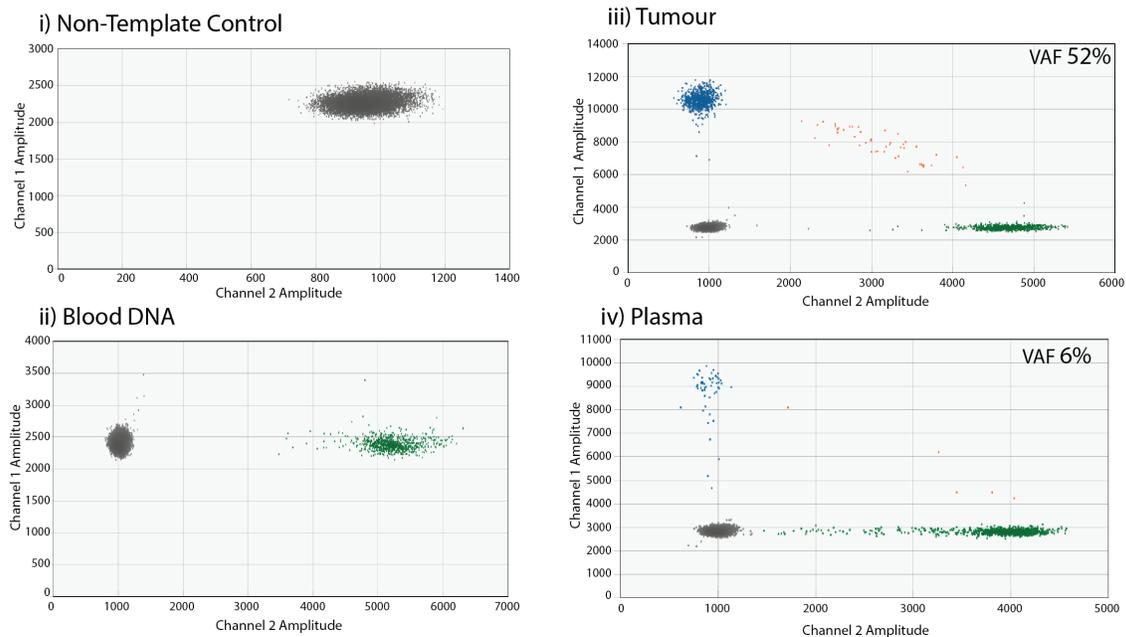


Fig. 3

Detection of tumour-specific alterations in sarcoma. Results for a representative case of localized extremity undifferentiated pleomorphic sarcoma. Whole exome sequencing of the primary tumour revealed a COL19A1-G125A variant. a) Results of a dilutional assay, demonstrating our ability to detect this tumour-specific variant at 0.5% of the wild type genome with droplet polymerase chain reaction (ddPCR). b) Results demonstrating that the COL19A1-G125A variant was detectable in the patient's plasma. Shown are ddPCR results for DNA extracted from the patient's 1) a non-template control, 2) whole blood (i.e. wild-type; 20 ng DNA), 3) tumour (10 ng DNA), and 4) plasma (i.e. circulating tumour DNA; 10 ng DNA). Green = wild-type-positive droplets; blue = variant-positive droplets; orange = variant/wild-type positive droplets; grey = negative droplets. VAF, variant allele frequency.

Detection of tumour-specific alterations in plasma has several theoretical advantages over cfDNA. Unlike cfDNA, which fluctuates physiologically in response to exercise,

cellular injury, and inflammation, ctDNA is tumour-specific. In addition, this biomarker provides insight into disease progression as tumour-specific alterations in

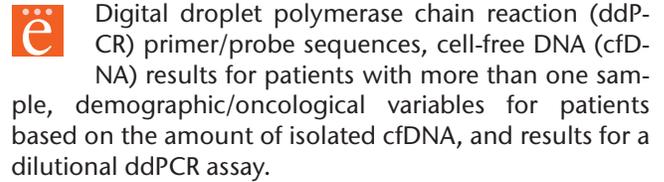
circulation may differ from those present in the primary tumour or in metastases. Measurement of ctDNA relies on a priori knowledge of allele specific mutations that can be used to identify DNA derived from a tumour cell in an abundant wild-type background. While this method is particularly promising in solid tumours such as lung and breast carcinomas, sarcomas present a unique challenge as the majority do not have recurrent genomic alterations that can be used for detection of ctDNA across multiple different cases. In this exploratory work, we demonstrate that ctDNA is detectable in the circulation of sarcoma patients by leveraging tumour-specific alterations present in the primary tumour to design custom primers and probes for ddPCR. Steps taken to decrease the likelihood of germline DNA contamination by blood cell lysis are important for prevention of further signal dilution, and we demonstrate that our cfDNA isolation protocol led to the ability to detect ctDNA in five of the six cases that were tested. Mutant alleles were detectable at a level of 0.25 ng of DNA or in 0.5% to 2.5% of the wildtype genome, the latter depending on variant allele frequency, and the fractional abundance of the mutant allele remained relatively constant even with low amounts of DNA. A number of other studies have successfully detected ctDNA in sarcoma, although the majority included only subtypes with known recurrent alterations, such as chondrosarcoma,¹⁷ myxoid liposarcoma,¹⁸ synovial sarcoma,¹⁸ and dedifferentiated liposarcoma.¹⁸ Another approach has been to use either commercially available or custom cancer gene panels.^{1,32–34} While this is more cost-effective and less labour-intensive, the ability to detect ctDNA appears to be inferior. For example, Demoret et al³³ used a commercial ctDNA panel in plasma taken from patients with metastatic leiomyosarcoma, undifferentiated pleomorphic sarcoma, dedifferentiated liposarcoma, and gastrointestinal stromal tumours, and were only able to detect panel-gene ctDNA in 18 of 24 cases.

This study has several limitations. As this was a pilot study, the patient cohort was small, which may lead to selection bias. Similarly, median follow-up was only 13.0 months (IQR 4.8 to 17.0). As the annual rate of development of metastases remains up to 10% until three years post-surgical resection,³⁵ this study may underestimate disease-free and overall survival. Finally, for the majority of patients, only a single sample was obtained. It is therefore unknown whether temporal changes in genomic alterations, in response to treatment or disease progression,^{36–39} will influence assay performance. A follow-up study based on the results of this exploratory work is underway and includes a larger number of patients, longer follow-up, and serial longitudinal sampling.

In conclusion, this pilot study evaluating the use of cfDNA and ctDNA in a range of bone and soft-tissue sarcomas shows promise for both biomarkers. Although these results must be confirmed using a larger patient cohort, we demonstrate that baseline quantification of plasma cfDNA in sarcoma patients is feasible and may have prognostic utility. We also show that our technique

permits detection of ctDNA even from specimens with low levels of DNA and for subtypes without recurrent genetic alterations. Future work will validate this approach in a larger number of cases, analyze cfDNA isolated from plasma longitudinally following resection of the primary tumour, and explore options for a multiplexed assay that is able to detect multiple tumour-specific alterations simultaneously. These steps are required to make more definitive assertions regarding the analytical and clinical utility of cfDNA and ctDNA in sarcoma.

Supplementary material

 Digital droplet polymerase chain reaction (ddPCR) primer/probe sequences, cell-free DNA (cfDNA) results for patients with more than one sample, demographic/oncological variables for patients based on the amount of isolated cfDNA, and results for a dilutional ddPCR assay.

References

1. Eastley NC, Ottolini B, Neumann R, et al. Circulating tumour-derived DNA in metastatic soft tissue sarcoma. *Oncotarget*. 2018;9(12):10549–10560.
2. Bagley SJ, Nabavizadeh SA, Mays JJ, et al. Clinical utility of plasma cell-free DNA in adult patients with newly diagnosed glioblastoma: A pilot prospective study. *Clin Cancer Res*. 2020;26(2):397–407.
3. Breitbach S, Sterzing B, Magallanes C, Tug S, Simon P. Direct measurement of cell-free DNA from serially collected capillary plasma during incremental exercise. *J Appl Physiol* (1985). 2014;117(2):119–130.
4. Vlamincck D. Noninvasive monitoring of infection and rejection after lung transplantation. *Proc Natl Acad Sci U S A*. 2015;112:13336–13341.
5. Kolesnikova E, Tamkovich SN, Bryzgunova OE, et al. Circulating DNA in the blood of gastric cancer patients. *Ann N Y Acad Sci*. 2008;1137:226–231.
6. Yoon KA, Park S, Lee SH, Kim JH, Lee JS. Comparison of circulating plasma DNA levels between lung cancer patients and healthy controls. *J Mol Diagn*. 2009;11(3):182–185.
7. Catarino R, Ferreira MM, Rodrigues H, et al. Quantification of free circulating tumor DNA as a diagnostic marker for breast cancer. *DNA Cell Biol*. 2008;27(8):415–421.
8. Swisher EM, Wollan M, Mahtani SM, et al. Tumor-specific p53 sequences in blood and peritoneal fluid of women with epithelial ovarian cancer. *Am J Obstet Gynecol*. 2005;193(3 Pt 1):662–667.
9. Kimura H. Detection of epidermal growth factor receptor mutations in serum as a predictor of the response to gefitinib in patients with non-small-cell lung cancer. *Clin Cancer Res*. 2006;12(13):3915–3921.
10. Miotke L, Lau BT, Rumma RT, Ji HP. High sensitivity detection and quantitation of DNA copy number and single nucleotide variants with single color droplet digital PCR. *Anal Chem*. 2014;86(5):2618–2624.
11. Borsu L. Clinical application of picodroplet digital PCR technology for rapid detection of EGFR T790M in next-generation sequencing libraries and DNA from limited tumor samples. *J Mol Diagn*. 2016;18(6):903–911.
12. Thompson JC, Yee SS, Troxel AB, et al. Detection of therapeutically targetable driver and resistance mutations in lung cancer patients by next-generation sequencing of cell-free circulating tumor DNA. *Clin Cancer Res*. 2016;22(23):5772–5782.
13. Valpione S. Plasma total cell-free DNA (cfDNA) is a surrogate biomarker for tumour burden and a prognostic biomarker for survival in metastatic melanoma patients. *Eur J Cancer*. 2018;88:1–9.
14. Lapin M, Oltedal S, Tjensvoll K, et al. Fragment size and level of cell-free DNA provide prognostic information in patients with advanced pancreatic cancer. *J Transl Med*. 2018;16(1):300.
15. Garland F, Laurent-Puig P, Sefrioui D, et al. Early evaluation of circulating tumor DNA as marker of therapeutic efficacy in metastatic colorectal cancer patients (PLACOL study). *Clin Cancer Res*. 2017;23(18):5416–5425.
16. Italiano A, Le Cesne A, Mendiboure J, et al. Prognostic factors and impact of adjuvant treatments on local and metastatic relapse of soft-tissue sarcoma patients in the competing risks setting. *Cancer*. 2014;120(21):3361–3369.

17. **Gutteridge A, Rathbone VM, Gibbons R, et al.** Digital PCR analysis of circulating tumor DNA: A biomarker for chondrosarcoma diagnosis, prognostication, and residual disease detection. *Cancer Med.* 2017;6(10):2194–2202.
18. **Braig D, Becherer C, Bickert C, et al.** Genotyping of circulating cell-free DNA enables noninvasive tumor detection in myxoid liposarcomas. *Int J Cancer.* 2019;145(4):1148–1161.
19. **Lewin J, Garg S, Lau BY, et al.** Identifying actionable variants using next generation sequencing in patients with a historical diagnosis of undifferentiated pleomorphic sarcoma. *Int J Cancer.* 2018;142(1):57–65.
20. **Yao W, Mei C, Nan X, Hui L.** Evaluation and comparison of in vitro degradation kinetics of DNA in serum, urine and saliva: A qualitative study. *Gene.* 2016;590(1):142–148.
21. **Cheng H, Liu C, Jiang J, et al.** Analysis of ctDNA to predict prognosis and monitor treatment responses in metastatic pancreatic cancer patients. *Int J Cancer.* 2017;140(10):2344–2350.
22. **Garcia-Murillas I, Schiavon G, Weigelt B, et al.** Mutation tracking in circulating tumor dna predicts relapse in early breast cancer. *Sci Transl Med.* 2015;7(302):302ra133.
23. **Namlos HM.** Use of liquid biopsies to monitor disease progression in a sarcoma patient: A case report. *BMC Cancer.* 2017;17:29–8.
24. **World Medical Association.** World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. *JAMA.* 2013;310(20):2191–2194.
25. **Takahashi G, Yamada T, Iwai T, et al.** Oncological assessment of stent placement for obstructive colorectal cancer from circulating cell-free DNA and circulating tumor dna dynamics. *Ann Surg Oncol.* 2018;25(3):737–744.
26. **Fan HC, Blumenfeld YJ, Chitkara U, Hudgins L, Quake SR.** Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. *Proc Natl Acad Sci U S A.* 2008;105(42):16266–16271.
27. **Stroun M, Lyautey J, Lederrey C, Olson-Sand A, Anker P.** About the possible origin and mechanism of circulating DNA apoptosis and active DNA release. *Clin Chim Acta.* 2001;313(1–2):139–142.
28. **Wan JCM, Massie C, Garcia-Corbacho J, et al.** Liquid biopsies come of age: towards implementation of circulating tumour DNA. *Nat Rev Cancer.* 2017;17(4):223–238.
29. **He S, Wang Y, Bao Y, et al.** Dynamic changes in plasma epstein-barr virus dna load during treatment have prognostic value in nasopharyngeal carcinoma: A retrospective study. *Cancer Med.* 2018;7(4):1110–1117.
30. **De Michino S, Aparnathi M, Rostami A, Lok BH, Bratman SV.** The utility of liquid biopsies in radiation oncology. *Int J Radiat Oncol Biol Phys.* 2020;107(5):873–886.
31. **Eisenhauer EA, Therasse P, Bogaerts J, et al.** New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer.* 2009;45(2):228–247.
32. **Shulman DS, Klega K, Imamovic-Tuco A, et al.** Detection of circulating tumour DNA is associated with inferior outcomes in Ewing sarcoma and osteosarcoma: a report from the Children's Oncology Group. *Br J Cancer.* 2018;119(5):615–621.
33. **Demoret B, Gregg J, Liebner DA, Tinoco G, Lenobel S, Chen JL.** Prospective Evaluation of the Concordance of Commercial Circulating Tumor DNA Alterations with Tumor-Based Sequencing across Multiple Soft Tissue Sarcoma Subtypes. *Cancers (Basel).* 2019;11(12):1829.
34. **Hemming ML, Klega KS, Rhoades J, et al.** Detection of Circulating Tumor DNA in Patients With Leiomyosarcoma With Progressive Disease. *JCO Precis Oncol.* 2019;2019:PO.18.00235.
35. **Wilson DAJ, Gazendam A, Visgauss J, et al.** Designing a rational follow-up schedule for patients with extremity soft tissue sarcoma. *Ann Surg Oncol.* 2020;27(6):2033–2041.
36. **Murtaza M, Dawson S-J, Tsui DWY, et al.** Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. *Nature.* 2013;497(7447):108–112.
37. **Siravegna G, Mussolin B, Buscarino M, et al.** Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. *Nat Med.* 2015;21(7):795–801.
38. **Hastings RK, Openshaw MR, Vazquez M, et al.** Longitudinal whole-exome sequencing of cell-free DNA for tracking the co-evolutionary tumor and immune evasion dynamics: Longitudinal data from a single patient. *Ann Oncol.* 2021;32(5):681–684.
39. **Carreira S, Romanel A, Goodall J, et al.** Tumor clone dynamics in lethal prostate cancer. *Sci Transl Med.* 2014;6(254):254ra125.

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