Genotyping of circulating cell-free DNA enables non-invasive tumor detection in myxoid liposarcomas

Short title: ctDNA enables tumor detection in myxoid liposarcomas

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ABSTRACT

Soft tissue sarcomas (STS) are rare tumors of mesenchymal origin. About 50% of patients with STS experience relapse and more than 30% will die within 10 years after diagnosis. In this study we investigated circulating free DNA (cfDNA) and tumor-specific genetic alterations therein (circulating tumor DNA, ctDNA) as diagnostic biomarkers. Plasma concentrations and fragmentation of cfDNA was analyzed with quantitative PCR. Patients with STS (n=64) had significantly higher plasma concentrations and increased fragmentation of cfDNA when compared to patients in complete remission (n=19) and healthy controls (n=41) (p<0.01 and p<0.001). Due to overlapping values between patients with STS and controls, the sensitivity and specificity of these assays is limited.

Sensitive assays to detect genomic alterations in cfDNA of synovial sarcomas (t(X;18)), myxoid liposarcomas (t(12;16) and TERT C228T promoter mutation) and well-differentiated/de-differentiated liposarcomas (MDM2 amplifications) were established. ctDNA was quantified in nine liposarcoma patients during the course of their treatment. Levels of breakpoint t(12;16) and TERT C228T ctDNA correlated with the clinical course and tumor burden in patients with myxoid liposarcomas (n=4). ctDNA could detect minimal residual disease and tumor recurrence. In contrast, detection of MDM2 amplifications was not sensitive enough to detect tumors in patients with well-differentiated/de-differentiated liposarcomas (n=5).

Genotyping of cfDNA for tumor specific genetic alterations is a feasible and promising approach for monitoring tumor activity in patients with myxoid liposarcomas. Detection of ctDNA during follow-up examinations despite negative standard imaging studies might warrant more sensitive imaging (e.g. PET-CT) or closer follow-up intervals to timely localize and treat recurrences.
NOVELTY & IMPACT STATEMENTS

Detection of recurrence in patients with myxoid liposarcomas is difficult, as metastatic disease occurs throughout the body. We show in this study that quantification of circulating tumor-DNA is a novel approach for monitoring tumor activity in patients with myxoid liposarcomas. The feasibility of this approach has been shown in carcinomas and few other STS. It enables timely, non-invasive detection of minimal residual disease and tumor recurrence and might therefore improve treatment.
INTRODUCTION

Soft tissue sarcomas (STS) are rare tumors of mesenchymal origin that account for ~1% of all malignancies. The fourth edition of the World Health Organization (WHO) Classification of Tumors of Soft Tissue and Bone includes over 70 histologic subtypes \(^1\). There are two main molecular sub-classes of STS. The first is characterized by sarcomas with no specific genetic alterations on a complex background of numerous chromosomal changes such as somatic mutations, intergene deletions, gene amplifications, reciprocal translocations, and complex karyotypes. This group includes, among others, de-differentiated and pleomorphic liposarcomas, undifferentiated pleomorphic sarcomas, leiomyosarcomas and fibrosarcomas. The second is characterized by specific genetic translocations on a background of relatively few additional chromosomal changes \(^2\). This group includes myxoid liposarcoma (MLS) and synovial sarcoma. In MLS the chromosomal translocations t(12;16)(q13;p16) and t(12;22)(q13;22q11-12) lead to fusion of the DDIT3 gene to FUS or EWS and in synovial sarcoma the t(X;18)(p11;q11) leads to fusion of the SS18 gene to SSX1, SSX2 or SSX4. The resulting fusion oncogenes have a causative role in the initiation and proliferation of these tumors \(^3\text{--}^5\). Hotspot mutations are rare in STS. However, undifferentiated pleomorphic sarcomas frequently harbor mutations in the TP53 gene and myxoid liposarcomas in the promoter of the TERT gene \(^6\text{,}^7\).

Complete resection with negative margins and radiotherapy represent the standard treatment strategy used for localized tumors. Still about 50% of patients with STS of the limbs experience relapse of any kind and more than 30% will die within 10 years after diagnosis \(^8\text{,}^9\). Most STS primarily metastasize to the lungs, although metastatic spread to extra-pulmonary sites is common in some STS types, especially MLS \(^10\). Detection of recurrence during follow-up is difficult and costly. Because there are no diagnostic biomarkers available, a thorough clinical examination and various imaging approaches taken at close intervals are typically used. Imaging is usually restricted to the lungs and the site of the primary tumor, as these are the most common locations for recurrence. Therefore, extra-pulmonary metastasis is frequently missed by imaging and only detected at advanced stages due to palpable lumps \(^11\).
Liquid biopsy might overcome many of these limitations, as it requires only a small blood specimen for noninvasive detection of tumor burden independent of its anatomic localization. Fragments of DNA are shed into the bloodstream by necrotic and apoptotic cells, and the load of circulating cell-free DNA (cfDNA) correlates with tumor staging and prognosis in many carcinomas. Genotyping of cfDNA for tumor specific mutations (circulating tumor DNA, ctDNA) has proven even more effective in tracking tumor dynamics. Quantification of ctDNA in the peripheral circulation might even surpass common imaging modalities in its sensitivity and specificity to detect tumors 12, 13. Besides monitoring tumor recurrence, ctDNA has been successfully implemented to detect Minimal Residual Disease (MRD) in patients with stage II colon cancer 14.

Establishing biomarkers in STS is difficult due to their rarity and the diverse nature. Here we investigate the utility of cfDNA analysis as a diagnostic biomarker in sarcomas. We further quantify ctDNA by targeting tumor-specific translocations and amplifications in a subset of soft-tissue sarcomas.
MATERIALS AND METHODS

Study population

Sarcoma patients were treated at the Comprehensive Cancer Center Freiburg. The study includes n=64 patients with active STS and n=19 patients with STS in complete remission. Healthy individuals (n=41) who had no history of cancer served as controls. A detailed description of the study population can be found in Supplementary Table 1.

Ethics, consent and permissions

The Ethics Committee of the Albert-Ludwigs-University of Freiburg, Germany, approved the study (study number: 236/16). The design and performance of the study is in accordance with the Declaration of Helsinki. Signed informed consent was obtained from all participants before inclusion, allowing analysis of tumor tissue, blood samples and clinical data.

Blood sampling

All blood samples were collected by puncture of the antecubital vein without tourniquet through a 20-gauge needle. The first 3 ml of blood were discarded. Each 9 ml of whole blood were collected in K$_2$EDTA (1.6 mg EDTA / ml blood) tubes (Sarstedt AG & Co, Nümbrecht, Germany) or in cfDNA tubes containing a cell-stabilizing additive (Roche Diagnostics, Basel, Switzerland). Blood was processed within 2 h after blood withdrawal, if not otherwise indicated. Blood samples were double centrifuged for 15 min at 2,000 g at 22 °C. Plasma aliquots were stored in cryos tubes (Greiner Bio-One GmbH, Frickenhausen, Germany) at -80°C before use.

Shipping of samples

Blood samples were collected into two K$_2$EDTA and two cfDNA tubes from each donor. One K$_2$EDTA tube and one cfDNA tube were processed within 2 h after blood withdrawal as described above. The second K$_2$EDTA tube and cfDNA tube from each donor were shipped by national mail to simulate the
following scenario: blood withdrawal at a distant location, shipping of the samples and processing at a specialized laboratory. The shipped blood samples were processed 72 h after withdrawal.

**Cell-free DNA isolation**

Cell-free DNA was extracted from 0.9 ml to 2 ml of plasma using the QIAamp Circulating DNA Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. An elution volume of 40 µl was used. Purified cfDNA was stored in low bind DNA tubes (Qiagen, Hilden, Germany) at -20°C.

**Quantification and integrity analysis of cell-free DNA**

Quantity and integrity of cell-free DNA circulating in plasma was evaluated by quantitative real-time PCR (qPCR) assays targeting the human β-globin and EIF2C1 genes. Further information can be found in Supplementary Methods.

**Amplification and sequencing of chromosomal breakpoints**

Genomic long-distance PCR was used to amplify the chromosomal breakpoints in myxoid liposarcomas and synovial sarcomas. The PCR was adapted from Wei et al. for synovial sarcoma fusion genes and Xiang et al. for myxoid liposarcoma fusion genes. The PCR products were gel-purified and sequenced by Sanger Sequencing (GATC Biotech AG, Köln, Germany). The nucleotide sequences were mapped against the human genome (GRCh38) with the Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov) to resolve the breakpoints at base pair resolution. Further details can be found in Supplementary Methods.

**Quantification of breakpoint DNA**
For the quantification of breakpoint DNA, assays were designed using Beacon Designer software (PREMIER Biosoft, Palo Alto, CA, USA). A fluorescent probe (20 - 30 nt) was placed directly on the breakpoint, targeting the sequences of both adjacent chromosomal regions. Corresponding primers amplifying a short sequence (80 - 140 bp) covering the breakpoint region were selected. Assay performance was validated by serially diluting tumor DNA from cell lines or native tumor tissue (0.001 to 10 percent) into human genomic DNA. Only assays that could detect at least 0.01 percent of tumor DNA and did not show false positive results with human genomic DNA were used to quantify circulating tumor DNA in patients’ plasma samples. In qPCR assays, absolute quantification of ctDNA was performed with the aid of a standard curve. In addition droplet digital PCR (ddPCR) was used to simultaneously quantify ctDNA and cfDNA without the aid of a standard curve. A detailed description can be found in the Supplementary Methods.

Quantification of TERT C228T promoter mutations

To detect the TERT C228T promoter mutations, the QX100 Droplet Digital PCR System (BioRad) was used. A commercially available assay that amplifies a 113 bp long sequence of the TERT promoter region was used (BioRad; UniqueAssayID: dHsaEXD72405942). Samples were prepared according to the manufacturer’s recommendations. The PCR was performed with the following cycling conditions: The enzyme activation took place at 95°C for 10 min. It was followed by 50 cycles of denaturation at 96°C for 30 s, annealing at 62°C for 30 s and extension at 62°C for 60 s. The enzyme deactivation took place at 98 °C for 10 min. Fluorescence within each droplet was subsequently determined by the QX100 droplet reader and data analyzed with QuantaSoft Analysis Software (BioRad).

Volume rendering of tumors

Tumor volume analysis was performed on image data from Magnetic Resonance Imaging (MRI) and Computer Tomography (CT) acquisitions. Images were segmented in 3D using the ITK-SNAP (Version 3.60) toolkit for semi-automatic segmentation. Tumors were pre-segmented using the built in region growing algorithm and, if necessary, manually delineated.
**Statistics**

Patients’ demographic data were compared using Mann–Whitney U test for independent samples. p-values were rounded to 3 significant digits; p-values below 0.05 were considered statistically significant. An unpaired t-test was performed to compare means of cfDNA plasma concentrations and means of cfDNA integrity indices. Comparison of cfDNA concentrations in EDTA and cfDNA tubes was analyzed by 1way ANOVA. Receiver operating characteristic curve (ROC curve) and nonlinear regression analysis were done using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA).
RESULTS

Preanalytical considerations

To determine the influence of sample shipping and cell stabilizing additives on the concentrations of cell-free DNA in plasma, venous blood from three healthy volunteers was drawn into standard EDTA collection tubes and tubes containing a cell-stabilizing additive (cfDNA tubes). One pair of tubes (1 x EDTA and 1 x cfDNA) was processed within two hours after blood withdrawal whereas a second pair of tubes (1 x EDTA and 1 x cfDNA) was shipped via the national postal system and processed after 72 hours. Cell-free DNA was isolated and quantified by real-time PCR. If samples were processed within two hours, the mean concentration of cell-free DNA was 276 ng ml\(^{-1}\) in the EDTA samples and 202 ng ml\(^{-1}\) in the samples containing the stabilizing additive. The difference between both groups was not statistically significant. Samples which were processed after shipping had a mean cfDNA concentration of 5554 ng ml\(^{-1}\) in the EDTA group and 240 ng ml\(^{-1}\) in the cfDNA tube group (Figure 1a). The difference between these two groups was highly statistically significant (p<0.01, 1way ANOVA).

Quantification of cfDNA in patient plasma

cfDNA concentrations in patient plasma were determined by real-time PCR with the ß-globin amplicon. All plasma samples fell within the range of the standard curve. The mean concentration of cfDNA in sarcoma patients (n=64) was 274 ng ml\(^{-1}\) (range 20.8 – 1485 ng ml\(^{-1}\)). The mean cfDNA concentration in the control group (n=41) was 67 ng ml\(^{-1}\) (range 2.1 – 288 ng ml\(^{-1}\)) and the group of patients in complete remission (n=19) had a mean concentration of 65 ng ml\(^{-1}\) (range 7.2 – 175 ng ml\(^{-1}\)). cfDNA in tumor patients was significantly elevated when compared to patients in remission and healthy controls (Figure 1 b) (p<0.01 and p<0.001, unpaired t-test). The diagnostic ability to discriminate tumor patients from patients in remission and healthy controls is displayed by the receiver operating characteristic curve (ROC) analysis (Figure 1 c). The Area Under the ROC (AUC) was 0.85 in both scenarios. cfDNA concentrations were not significantly different between histological types of sarcomas, or localized and metastatic disease (Supplemental Figures 1 e + f).
Integrity of cell-free DNA

To determine the integrity of cfDNA we measured the ratio of longer (236 bp EIF2C1 amplicons) to shorter (82 bp EIF2C1 amplicons) DNA fragments. Both primer pairs amplified a single fragment of human DNA, which corresponded to its predicted size on an agarose gel (Figure 2 a). Assuming equal efficiency in the amplification of both fragments, the DNA integrity for unfragmented DNA would approximate 1. If the assay was performed with unfragmented human genomic DNA, it yielded a mean DNA integrity index of 0.79 (SD: 0.08). Cell-free DNA from healthy controls (n=41) yielded a DNA integrity of 0.21 (range 0.11 – 0.42) and that from patients in complete remission (n=19) was 0.29 (range 0.12 – 0.49). The mean cfDNA integrity of tumor patients (n=64) was 0.16 (range 0.06 – 0.33) and thus significantly less than in the two former groups (Unpaired t-test, p< 0.001) (Figure 2 b). ROC curve analysis comparing the tumor patients to the healthy controls showed an AUC of 0.73 and an AUC of 0.85 comparing the tumor patients’ group to the complete remission group (Figure 2 c).

Genotyping of copy number variations

Ring chromosomes, allowing for gene amplification, constitute the characteristic cytogenetic feature of some STS including well-differentiated and de-differentiated liposarcoma (WDLS/DDLS). They contain multiple copies of the target genes CDK4, HMGA2 and MDM2 from 12q. We developed a ddPCR assay to detect MDM2 copy number variations (CNV) at low frequency. One primer pair amplifies a 70 bp region in MDM2, which is detected by a FAM labelled probe. A reference gene (EIF2C1), which is not amplified in STS, is detected by a HEX labelled probe. Wild type DNA, which contains equal amounts of both genes thus yields a quotient of MDM2/EIF2C1 of 1. In contrast, genomic DNA from the WDLS cell line T778, which carries a known amplification of the MDM2 gene, results in a MDM2/EIF2C1 quotient of 32 (Supplemental Figure 2 a). We determined the detection limit of the assay by serially diluting T778 genomic DNA into wild type DNA. 5% T778 DNA revealed a MDM2/EIF2C1 quotient of 1.2, which visibly differed from the baseline. In contrast, we could not reliably distinguish a 0.5 % T778 DNA fraction from wild type only DNA (Supplemental Figure 2 b).
We then analyzed cfDNA of n=5 patients with WDLS/DDLS for MDM2 copy number variations. All tumors had MDM2 amplifications, as proven by CISH. cfDNA of WDLS/DDLS patients had a mean MDM2{EIF2C1 ratio of 1.21 (range 1.14 – 1.38), whereas healthy controls (n=19) had a mean MDM2{EIF2C1 ratio of 1.09 (range 0.69 - 1.41) (Supplemental Figure 2 c). There was no statistically significant difference between both groups (Unpaired t-test, p=0.14).

Quantification of recurrent sarcoma translocations in cfDNA

We targeted the recurrent chromosomal translocation t(X;18) in two synovial sarcoma cell-lines and t(12;16) in two myxoid liposarcoma cell-lines and 4 patients with myxoid liposarcomas. In synovial sarcoma the translocation occurs between the SS18 gene and either the SSX1or SSX2 gene, or in rare events the SSX4 gene. In MLS the chromosomal translocations lead to fusion of the DDIT3 gene to FUS or EWS. The SS18-SSX1 and SS18-SSX2 as well as FUS-DDIT3 breakpoints were amplified by long-range PCR from chromosomal DNA of cell lines harboring the respective translocations. The primers specifically amplified the targeted breakpoint regions (Figure 3 a). Breakpoint amplicons were sequenced by Sanger sequencing and the nucleotide sequences were mapped to the human genome (GRCh38/hg38) to decode the breakpoints at base pair resolution (Figure 3 b and Supplemental Figure 3). Quantitative real-time PCR assays were designed, which specifically amplify a small fragment covering the breakpoint region with the fluorescent probe placed directly over the breakpoint (Figure 3 b). These assays could quantify tumor DNA in a linear range from 0.024 ng to 87 ng when spiked into 132 ng of fragmented human wild type DNA. The detection limit of ctDNA thus corresponds to 4 genome equivalents and a fraction of only 0.01 % (Figure 3 c). The same assays can also be run on a ddPCR system, which allows direct quantification of ctDNA without the aid of a standard curve (Figure 3 d). Including a wild type amplicon (EIF2C1_82) in the ddPCR assay enables the simultaneous quantification of cfDNA and ctDNA in the sample (Figure 3 e).

Due to the balanced nature of the translocation, a second breakpoint exists in addition to the biological driver translocation. We therefore designed primers to also amplify the reciprocal breakpoints in MLS.
Due to different amplification efficiencies of the amplicons, droplets arising from the regular and reciprocal breakpoints can be distinguished by multiplex ddPCR, despite using two FAM-labelled probes. This allows simultaneous quantification of both breakpoints within a single sample and thus increases the chance to detect ctDNA (Figure 3 f).

Quantification of TERT C228T promoter mutation

TERT promoter mutations frequently occur in myxoid liposarcomas, but rarely in other soft-tissue sarcomas. They are believed to play a pivotal role in disease progression as a secondary genetic event to the initiating t(12;16) translocation when tumor cells face the need for telomere elongation to allow further proliferation.

The TERT ddPCR assay reliably separated TERT wild type from TERT C228T mutant droplets (Figure 4 a). We determined the sensitivity of the assay by spiking increasing amounts of TERT C228T mutant DNA (MLS 402) into human wild type DNA. The number of measured mutant droplets correlated well with the calculated values at a relative fraction of > 0.5 - 1 per cent of TERT C228T mutant DNA. It was able to detect relative fractions < 0.5 per cent mutant DNA, however tended to overestimate the absolute amounts (Figure 4 b). False positive mutants droplets might occur in this assay, as the fluorescent probes, which distinguish between the mutant and wild type allele differ by only one base. As this event might occur more frequently with increasing DNA fragments, we determined the false positive mutant droplets dependent on the absolute amount of wild type DNA in the assay. Assays were performed in duplicates and only 3 out of 14 wells showed false positive C228T mutant droplets at low frequency. We did not observe increasing false positive rates with increasing amounts of wild type DNA (Figure 4 c). We subsequently determined the false positive rate of the assay with cfDNA from healthy controls (n=12). The assay demonstrated a mean false positive rate of 12 mutant copies ml⁻¹ plasma, ranging from 0 - 28 mutant copies ml⁻¹ plasma (Figure 4 d).

Detection of ctDNA in patient samples

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Quantification of ctDNA was performed in four patients with myxoid liposarcomas during the course of treatment. A patient (MLS 8811) presented with a large primary MLS of the left thigh with a tumor volume of 927 cm³ (Figure 5a). Further staging revealed no evidence of metastatic spread and the tumor was subsequently excised with negative margins. A follow-up examination of the primary site with magnetic resonance imaging (MRI) and the lungs with computed tomography (CT) after three months showed no signs of recurrence. However, several small metastases of both lungs with a combined volume of 7.7 cm³ became evident at the 6 months follow-up examination. This suggests that MRD was still present when the primary tumor was removed but could not be detected by CT.

Subsequently all metastases from the left lung were surgically removed (R0), leaving behind a small residual tumor of 0.24 cm³ in the right lung. The patient declined further surgery and was lost to follow-up after discharge from the hospital. We obtained tissue from the primary tumor and a total of n=7 blood samples during the course of treatment. The primary tumor harboured the TERT C228T promoter mutation (Supplemental Figure 4a). Both breakpoints were amplified and sequenced from the tumor, which showed a balanced translocation t(12;16) (Figure 5b and Supplemental Figure 5a). We designed and validated ctDNA assays for both breakpoints. The regular breakpoint assay was able to detect ctDNA at a detection limit of 4 copies/well and a fraction of 0.01 % and showed no false-positives for wild type only DNA (Figure 5b). We could not design an assay for the reciprocal translocation due to genome-repetitive DNA sequences at the flanking regions of the breakpoint at the binding sites of potential primers.

cfDNA was extracted from the plasma samples and analyzed by the ctDNA breakpoint assay (Fig 5c). The concentration of ctDNA correlated with the clinical course of the disease. ctDNA rapidly decreased from 175 copies ml⁻¹ to 18 copies ml⁻¹ 2 weeks after resection of the primary tumor, but did not reach baseline. The remaining ctDNA might reflect MRD which did not become evident during the initial staging examinations. Whereas MRI/CT staging 3 months after tumor resection still suggested complete remission, ctDNA concentrations were again elevated (121 copies ml⁻¹). ctDNA concentrations rose concomitantly with the growing tumor burden in both lungs and reached 281
copies ml⁻¹ one day before lung surgery. Partial resection of lung metastases again led to a decline of ctDNA in the peripheral circulation to 29 copies ml⁻¹ two weeks after surgery, again remaining above the detection limit. Except for the early postoperative samples, cfDNA concentrations paralleled the course of ctDNA. ctDNA constituted only a fraction of 0.25 percent to 6.4 percent of the cfDNA (Figure 5 c).

Genotyping of the cfDNA samples for the TERT C228T mutations showed similar concentrations and an analogue course of the TERT C228T ctDNA when compared to the t(12;16) ctDNA (Figure 5 d and Supplemental Figure 4 b). ctDNA concentrations ranged from 60 to 244 copies ml⁻¹ and were thus well above the false positive rate of the assay.

We further validated our approach in a second patient with a localized MLS (MLS 3910) of the right pelvis (tumor volume: 104 cm³). Again, the FUS/DDIT3 breakpoints were amplified and sequenced from native tumor tissue and tumor specific qPCR breakpoint assay were designed and validated as described above (Figure 6 a and Supplemental Figure 5 b). The assay for the regular breakpoint was able to detect ctDNA at a detection limit of 4 copies/well and a fraction of 0.01 % and showed no false-positives for wild type only DNA (Figure 6 b). In this patient we could also design a qPCR assay for the reciprocal breakpoint. However, the detection limit of the assay was inferior to the regular breakpoint assay (detection limit of 22 copies/well and a fraction of 0,1%; Supplemental Figure 5 c).

With the assay for the regular breakpoint we detected 33 copies ml⁻¹ of ctDNA in the first sample, which was taken preoperatively during neoadjuvant radiotherapy. ctDNA values dropped beneath the detection limit of the assay 10 days after tumor resection. Whereas ctDNA levels reflected the clinical course, cfDNA values markedly rose in the early postoperative period. ctDNA constituted a fraction of 0.13 percent of the cfDNA in the preoperative sample (Figure 6 c).

The patient developed metastatic disease 1.5 years later with several bone metastases of the spine and femur, which were treated by radiotherapy. In the latter course a mediastinal metastasis was detected and treated by surgery and adjuvant radiotherapy, which achieved good local control. However, 2 months later, several large retroperitoneal metastases were discovered. The patient
declined further treatment and received best supportive care. We obtained 4 plasma samples during the course of his treatment.

tcDNA concentrations were detectable at 67 copies ml⁻¹ (sample 1) after the bone metastasis had been treated with radiotherapy. They declined to 26 copies ml⁻¹ (sample 2) when the mediastinal tumor was detected. We could not detect ctDNA in the third sample, which was obtained after removal of the mediastinal mass during adjuvant radiotherapy. The concentration rose once more to 292 copies ml⁻¹ (sample 4) when the patient developed disseminated retroperitoneal metastases (Figure 6 d). cfDNA concentrations rose during the course of radiotherapy and increased further upon development of disseminated metastatic spread. ctDNA comprised a fraction of 0 to 0.80 percent of the cfDNA (Figure 6 d).

The assay for the reciprocal breakpoint also detected ctDNA in most samples. In contrast to the regular breakpoint assay, it could not detect ctDNA in sample 2 (Supplemental Figure 5 d).

We included two additional patients with localized myxoid liposarcomas in the study. A patient (MLS 4840) presented with a deep-seated, localized MLS of the right upper thigh (tumor volume: 82 cm³), which was completely removed with negative margins. Imaging with MRI of the primary site and CT of the lungs indicated complete remission 10 months after surgery. t(12;16) and TERT C228T ctDNA was quantified at similar concentrations in the preoperative samples with no detectable ctDNA at the follow-up examination (Figure 6 e). Cell-free DNA concentrations were low compared to other patients and marginally decreased after tumor removal. ctDNA constituted a fraction of 4 - 5 per cent of the cfDNA in the preoperative sample.

Another patient (MLS 2826) also suffered from a localized tumor of the upper thigh (tumor volume: 56 cm³), which was surgically removed. We obtained three plasma samples during the course of his treatment. Genotyping of cfDNA was performed with the TERT C228T assay. ctDNA reflected the clinical course of his disease. It decreased from 640 copies ml⁻¹ to 304 copies ml⁻¹ within days after tumor removal and within the false-positive range 104 days after tumor excision (9 copies ml⁻¹). cfDNA levels again peaked in the early postoperative period but otherwise paralleled the ctDNA levels.
Although we were able to sequence the t(12;16) breakpoint, the resultant patient-specific assay was not able to reliably detect t(12;16) DNA with high sensitivity and specificity.
DISCUSSION

Establishing tumor markers in STS is difficult because of their rarity and genetic diversity. Circulating DNA holds the strong potential to improve and simplify cancer detection and treatment prediction. To date it has mostly been adapted to epithelial cancers, most commonly lung, breast, and colon cancer\textsuperscript{20}. Here we show that cfDNA can also be used as a diagnostic biomarker in soft tissue sarcomas. We demonstrate that quantification and fragmentation analysis of cfDNA can separate patients from healthy controls and patients in complete remission. We further prove that genotyping of cfDNA for sarcoma specific alterations enables sensitive detection of individual tumors in the peripheral blood of patients with myxoid liposarcomas. More specifically, absolute quantification of ctDNA correlates with the tumor burden and can be used to detect recurrence and potentially minimal residual disease.

Quantification and fragmentation analysis can be performed without prior knowledge of the histological subtype or tumor specific mutations. This is of great advantage in STS, which are comprised of more than 70 types with different pathological and clinical features, as well as a diverse range of genetic alterations\textsuperscript{6}. Our data shows a sensitivity of 80\% and a specificity of 68\% for cfDNA quantification and a sensitivity of 79\% and a specificity of 73\% for cfDNA integrity testing to discriminate tumor patients from patients in remission. This is in agreement with studies in other cancers that show a similar area under the receiver operating characteristic curve to discriminate patients from controls by quantification and integrity testing of cfDNA. Additionally, plasma concentrations in these cohorts were similar to those of our samples where quantification was performed by qPCR\textsuperscript{15,21}. Still, as these methods do not detect specific genetic events, they are prone to preanalytical errors. Exercise, medical conditions, surgery, and trauma can all increase plasma levels of cfDNA and thereby interfere with assay performance. In addition, sample handling, shipping, and time to cfDNA purification can increase cell-free DNA levels due to leukocyte disintegration\textsuperscript{13}. Although using specific cfDNA tubes can compensate for the latter, the confounding factors mentioned above combined with the limited sensitivity and specificity of the assays will probably preclude its widespread clinical use.
Genotyping of cfDNA offers the advantage of detecting tumor specific genetic alterations in the peripheral blood. It therefore circumvents most of the limitations mentioned above and enables tumor detection to surpass radiographic approaches in sensitivity. We demonstrate that this approach is also applicable to myxoid sarcomas as levels of ctDNA correlated with tumor volume and tumor stage. Sarcomas with simple karyotypes harbouring a defined translocation, like synovial sarcomas, myxoid liposarcomas, and Ewing sarcomas in particular offer the possibility for a targeted approach with highly sensitive and specific quantification of breakpoint DNA in the peripheral circulation. As amplification of the target region is performed with primers binding to separate chromosomal regions and detection of the target amplicon occurs by a probe that is placed on the breakpoint region, these assays offer unprecedented specificity. The feasibility of this approach has also recently been confirmed in Ewing sarcomas. The assays to detect synovial sarcoma t(X;18) ctDNA from cell-lines showed similar sensitivity and specificity than with myxoid liposarcomas. Unfortunately, we had native tumor tissue from only two synovial sarcoma patients, who had received radiation therapy before tumor resection. It turned out, that the content of tumor DNA in the necrotic tissue was too low to amplify the breakpoints with long-distance PCR. This is the prerequisite for the development of a sensitive personalized assay to detect ctDNA. Massive parallel sequencing based assays might overcome this problem and enable the detection of tumor-associated translocations even in samples with minimal amounts of tumor tissue.

In contrast, detection of copy number variation in peripheral blood has a lower sensitivity (ctDNA burden > 1-5%), as ctDNA is only indirectly quantified by assessing the amplified gene relative to a reference gene. Additionally, detection depends substantially on the rate of amplification, which is variable in DDLS and WDLS. As relative ctDNA concentrations usually constitute less than one percent in STS patients, CNV detection seems unsatisfactory to quantify ctDNA in STS patients with high sensitivity and specificity. To overcome these limitations, shallow whole genome sequencing might be used to detect concomitant structural variants at base-pair resolution, which might then be targeted by more sensitive approaches.
As sensitivity of ctDNA detection not only depends on assay performance, but also on the number of template molecules in the sample, detection of multiple mutations can greatly improve the detection threshold\textsuperscript{26}. We therefore started to sequence both breakpoints in MLS with balanced translocations and developed assays that simultaneously detected both target regions. These modified assays improved the detection rate of ctDNA by a factor of two. Simultaneous detection of multiple genetic alterations by highly sensitive and specific assays can be used to monitor early recurrence or minimal residual disease after complete resection of a localized tumor\textsuperscript{14}. This could be accomplished in sarcomas by adding recurrently mutated genes to the breakpoint assays\textsuperscript{7}. Deep sequencing of these multi-gene panels by massive parallel sequencing would thus allow an unprecedented sensitivity.

A major drawback of our breakpoint assays is the need for fresh frozen tumor tissue and the time consuming development of individualized patient assays. As each tumor harbours a unique breakpoint sequence, every breakpoint must be amplified and sequenced individually, and an assay must be designed and validated with this specific sequence. Targeted massive parallel sequencing of the breakpoint regions might overcome these limitations and allow breakpoint sequencing from FFPE tumor samples or directly from plasma samples. Maybe a combinatorial approach of massive parallel sequencing to detect multiple targetable mutations in conjunction with individual, highly sensitive assays to quantify ctDNA might provide the best performance\textsuperscript{24,27}.

Another possibility to circumvent the need of prior mutational analysis of tumor tissue is targeting of hotspot mutations by ddPCR. Unfortunately, hotspot mutations like the recurrent TERT promoter mutations, which can be found in approximately 75\% of MLS patients, are not existent in other sarcomas at similar frequency\textsuperscript{6,7}. Hotspot mutations offer the additional advantage that the same assay can be used for all tumors, which harbour the respective mutation. As expected, our TERT C228T ddPCR assay was able to detect ctDNA in most MLS patients at high sensitivity. However, due to the single nucleotide exchange only, its specificity was inferior to the t(12;16) breakpoint assays.

Given the intrinsic advantages and disadvantages of both assay types, quantification of ctDNA in MLS patients is best performed with both techniques to increase detection rates.
Detection of breakpoint DNA and hotspot mutations is limited to approximately 50 per cent of STS. The other half is characterized by no specific genetic alterations on a complex background of numerous chromosomal changes. These STS are not amenable to a targeted approach of a small genomic region, as presented in this study. However, a combinatorial approach that integrates different sequencing protocols to detect single-nucleotide variants, small indels, and copy-number alterations across a large genomic area might provide a suitable approach. Alternatively, a step-wise approach with mutation detection by shallow whole-genome or exome sequencing in tumor tissue with subsequent targeted mutation detection by deep sequencing in plasma could perform well over multiple STS subtypes.

In addition to cfDNA genotyping, other possibilities for non-invasive cancer detection for STS exist. Blood borne miRNA signatures were found to correlate with tumor activity in synovial sarcomas and rhabdomyosarcomas and several studies have detected circulating tumor cells in sarcoma patients. Whether any of these biomarkers is superior to ctDNA detection remains unresolved, and further studies are needed to independently validate and compare these markers.

Integrating non-invasive cancer detection into the diagnostic portfolio for myxoid liposarcoma patients offers multiple new possibilities to individually guide treatment. Patients with detectable ctDNA after complete tumor resection might be candidates for adjuvant systemic treatment, whereas ctDNA negative patients might be spared from the additional toxicity. In a different scenario, patients with a sudden rise in ctDNA might profit from more extensive imaging to adequately detect and treat tumor recurrence, whereas imaging in ctDNA negative patients might be reduced to spare radiation dose and costs.

Genotyping of cfDNA for tumor specific genetic alterations can be used to monitor tumor activity in myxoid liposarcomas. Quantification of ctDNA provides a powerful diagnostic tool to detect minimal residual disease and tumor recurrence in patients after primary treatment of localized tumors. It might
thus also help to guide personalized treatment in the era of precision medicine in patients with other soft tissue sarcomas.
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ABBREVIATIONS

STS, soft tissue sarcoma; cfDNA, free circulating DNA; ctDNA, circulating tumor DNA; MLS, myxoid liposarcoma; MRD, minimal residual disease; ddPCR, droplet digital PCR; CNV, copy number variations; WDLS, well differentiated liposarcoma; DDLS, dedifferentiated liposarcoma

AUTHORS’ CONTRIBUTIONS

David Braig: study design; sample acquisition; data acquisition; data analysis and interpretation; drafting of manuscript

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Christiane Bickert: data acquisition and analysis

Moritz Braig: analysis of MRI data; revision of manuscript

Rainer Claus: study design; data interpretation; revision of manuscript

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CONFLICT OF INTERESTS

D.B. received free trial Cell-Free DNA Collection Tubes from Roche Holding AG, Basel, Switzerland.

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REFERENCES


LEGENDS TO FIGURES

Figure 1: (a) Impact of blood stabilizing reagents on cell-free plasma DNA concentrations. Blood from three healthy volunteers was drawn in EDTA tubes and cfDNA tubes. Plasma was either obtained after 2 h or 72 h. Plasma DNA was isolated and quantified by qPCR. There was no significant difference in the amount of cfDNA isolated from EDTA or cfDNA tubes, if plasma was extracted after 2 h. Cell-free plasma DNA was elevated by a factor of x 20 in EDTA tubes, if samples were extracted after 72 h (p<0.01, 1way ANOVA). Displayed are means and SEM. All samples were analyzed in triplicate. (b) cfDNA concentrations are elevated in tumor patients. cfDNA from n=64 tumor patients, n=19 patients in complete remission and n=41 healthy controls was isolated and quantified. cfDNA in tumor patients was significantly elevated when compared to patients in remission and healthy controls (p<0.01 and p<0.001, unpaired t-test). Results are displayed in a box plot with whiskers from the 10th – 90th percentile. All samples were analyzed in triplicates. (c) The diagnostic ability to discriminate tumor patients from patients in remission and healthy controls is displayed by the receiver operating characteristic curve (ROC) analysis. Sensitivity is plotted on the y-axis and 1 – specificity on the x-axis. The Area Under the ROC (AUC) was 0.85 in both scenarios.
Figure 2: (a) cfDNA fragmentation was measured by calculating an integrity index from the amount of a longer (236 bp) EIF2C1 amplicon divided by a shorter (82 bp) EIF2C1 amplicon. The sequence from the shorter amplicon lies within the longer amplicon. The integrity index approximates 1 for unfragmented DNA and 0 with increasing degradation of DNA fragments. The agarose gel shows the specificity of both primer pairs, which specifically amplified both target sequences from human DNA. (b) cfDNA fragments are shorter in tumor patients. cfDNA integrity index from n=64 tumor patients, n=19 patients in complete remission and n=41 healthy controls was determined. DNA integrity was significantly reduced in tumor patients when compared to patients in remission and healthy controls (p<0.001, unpaired t-test). Results are displayed in a box plot with whiskers from the 10th – 90th percentile. All samples were analyzed in triplicates. (c) The diagnostic ability to discriminate tumor patients from patients in remission and healthy controls is displayed by receiver operating characteristic curve (ROC) analysis. The sensitivity is plotted on the y-axis and 1 − specificity on the x-axis. The Area Under the ROC (AUC) was 0.85 comparing tumor patients to patients in remission, and 0.73 comparing tumor patients to healthy controls.
Figure 3: Assays to detect breakpoint DNA from synovial sarcomas and myxoid liposarcomas.

(a) The genomic breakpoints from two myxoid liposarcomas (MLS 402, MLS 1765) with a t(12;16) translocation and two synovial sarcomas with t(X;18) were amplified by long range PCR from unfragmented genomic tumor DNA. DNA was separated on a 0.8 % agarose gel. (b) Shows the breakpoint (arrow) nucleotide sequence of the SS18/SSX1 translocation in the synovial sarcoma cell line HS-SYII. Primers (half arrows) to amplify the breakpoint sequence and the respective probe (blue) for the ctDNA assay are indicated. (c) Decreasing amounts of HS-SYII DNA was spiked into human wild type DNA and its amount determined by breakpoint qPCR assay. A strong linear correlation between the C_T values and the log of the template DNA (R^2 > 0.99) was observed. (d) t (X;18) breakpoint ctDNA was quantified by ddPCR. Decreasing amounts of HS-SYII DNA was spiked into wild type DNA and quantified by the breakpoint assay on a ddPCR instrument. Fractions of 0.1% could reliably be detected. A FAM-labelled probe detected the breakpoint fragments (blue droplets). (e) Including a reference amplicon (EIF2C1) in the ddPCR assay that is detected by a HEX labelled probe (green droplets) allowed simultaneous quantification of cfDNA. (f) Simultaneous detection of the regular and reciprocal breakpoint increases sensitivity. Both t(12;16) breakpoints from a myxoid liposarcoma (MLS 402) were sequenced and breakpoint specific assays established as described under (b). Both probes were FAM-labelled. MLS 402 DNA was spiked into wild type DNA and quantified by ddPCR (blue droplets). Due to different amplification efficiencies of the assays, droplets from the regular and reciprocal breakpoint could be distinguished in a multiplex assay despite both being FAM labelled. A multiplex assay with both amplicons increased the sensitivity by a factor of 2.
Figure 4: ddPCR assay to detect the TERT C228T hotspot mutation in MLS. (a) A 113 bp fragment of the TERT promoter region is amplified within each droplet and probes with different fluorophores are used to detect C228T mutant (blue) and wild type (green) sequences. Droplets that harbor a mutant and wild type allele display a mixed signal (orange). Depicted are representative graphs with human genomic DNA and a mixture with a 1 per cent fraction of TERT C228T mutant DNA (MLS 402). (b) Increasing amounts of MLS 402 DNA were mixed into human genomic DNA and analyzed with the TERT C228T ddPCR assay. The measured and calculated TERT C228T copy numbers were plotted on a logarithmic scale. (c) The false-positive rate of the assay was determined by analyzing increasing amounts of human genomic DNA. Each concentration was analyzed in duplicates. (d) cfDNA was isolated from plasma of healthy controls (n=12) and analyzed with the TERT C228T ddPCR assay. Displayed are the false-positive mutant copies ml$^{-1}$. We observed a mean of 12 false positive copies ml$^{-1}$ (range: 0 - 28 copies ml$^{-1}$). Displayed are the individual concentrations, the mean and SD.
Figure 5: Circulating tumor DNA can detect recurrence and MRD in myxoid liposarcoma patients. (a) A patient presented with a large MLS of the left thigh. Preoperative staging revealed no signs of metastases and the tumor was completely resected. Follow-up of the local site by MRI and the lungs by CT revealed no signs of recurrence at 3 months after the operation. Several small metastases of both lungs were detected at 6 months follow-up. All but one metastasis with a volume of 0.24 cm³ could be surgically removed. Depicted are the MRI imaging of the primary tumor, the volume rendering of the primary tumor, a plane from the chest CT showing a lung metastasis and the clinical course with the calculated tumor volumes from MRI and CT imaging. (b) The t(12;16) breakpoint was amplified and sequenced from the primary tumor. A breakpoint specific assay was designed and validated as described in Figure 4. Again, a strong linear correlation between the Ct values and the log of the tumor DNA (R² > 0.99) was observed. The agarose gel depicts the 131 bp breakpoint amplicon from the assay, which only forms in the presence of tumor DNA. (c) We obtained 7 blood samples during the course of treatment. cfDNA was purified and quantified. t(12;16) ctDNA was quantified with the breakpoint assay. ctDNA was detectable at 175 copies ml⁻¹ plasma 1 day before tumor resection. It rapidly decreased postoperatively, but was still detectable at 18 copies ml⁻¹ 17 days after tumor resection. It was elevated at 121 copies ml⁻¹ at 3 months follow-up, when imaging still suggested complete remission and peaked at 281 copies ml⁻¹ before removal of the lung metastases. ctDNA again declined to 29 copies ml⁻¹ two weeks after surgery. cfDNA values paralleled the ctDNA levels except in the early postoperative period. ctDNA comprised a fraction of 0.25 - 6.4% of cfDNA (note the different scale of the right and left y-axis). ctDNA is depicted by black triangles and cfDNA by black squares. Values are the mean of triplicates. (d) ctDNA was quantified by the TERT C228T ddPCR assay. Concentrations paralleled those of the t(12;16) breakpoint assay and were well above the false-positive rate of the assay. The graph shows the results of the ddPCR assay of the sample that was taken 1 day before the first operation (8811-1). C228T mutant droplets are depicted in blue and wild type droplets in green.
Figure 6: Circulating tumor DNA can be used to monitor treatment in myxoid liposarcoma patients. (a) A MLS with a volume of 104 cm³ of the right pelvis was resected. (b) The t(12;16) breakpoint was sequenced and an individual assay designed. Depicted are the standard curve and the breakpoint amplicon as described in Figure 4 and 5. (c) t(12;16) tumor DNA was detectable preoperatively at 33 copies ml⁻¹ and rapidly declined below the detection limit of the assay within 10 days (black triangles). In contrast, cfDNA values increased by a factor of 7.5 (black squares). ctDNA comprised a fraction of 0.1% of cfDNA. (d) The patient later developed metastatic disease and we analyzed 4 samples during his treatment. The first sample was obtained after several bone metastases of the spine and femur were treated by radiotherapy. The second sample was taken when imaging showed a mediastinal mass, which was subsequently surgically removed. The third sample was taken during adjuvant radiotherapy. He then developed disseminated metastases (sample 4) and declined further treatment. Depicted are the ctDNA (black triangles) and the cfDNA values (black squares). ctDNA comprised a fraction of 0 to 0.80% of the cfDNA. (e) The tumor of a patient with a localized myxoid liposarcoma was completely excised and plasma samples were taken 1 day preoperatively and 317 days after tumor removal. Imaging at the follow-up examination showed no signs of tumor recurrence. t(12;16) and TERT C228T ctDNA was detectable before the operation but below the limit of detection at 317 days follow-up. (f) Three plasma samples of a patient with a myxoid liposarcoma were analysed during the course of his treatment. TERT C228T ctDNA rapidly decreased after tumor removal and converged towards the baseline at 104 days follow-up, where he was free of detectable recurrence. cfDNA peaked in the early postoperative period, but otherwise paralleled the ctDNA levels.
Figure 1

(a) Box plots showing cDNA (ng/ml) across different conditions: EDTA-0h, EDTA-24h, EDTA-72h, and cDNA-0h. The p-values are indicated as follows: p < 0.01, p < 0.01, n.s., n.s.

(b) Box plots showing cDNA (ng/ml) across different groups: tumor (n=6), remission (n=19), control (n=41). The p-values are indicated as p < 0.01, p < 0.001.

(c) ROC curves for tumor vs. remission and tumor vs. control, both with an AUC of 0.85.
Figure 3

a) 

b) HS-SY-II t(X;18)

Chr X (SSX1)

TTGGAGCTCTGGATAGACTACCACGCGACT

GTCGCCGAGAAATCTTATTAACTCTCTGTAAC

CGCGAAATGACAACCTTTCTCTCTAGATACAG

GAACAAGACAAAATGTCTACTCTCAACCATT

Chr 18 (SS18)

c) HS-SY-II DNA (ng)

ct values

d) 10% 1% 0.1% 0.0% 

Event Number

Ch1 Amplitude

e) t(X;18)

E2C1

f) MLS 402

t(12;16)

MLC 402

reciprocal t(12;16)

MLC 402

t(12;16) + reciprocal t(12;16)
Figure 5

a) MLS of left thigh

Resection of primary tumor → Resection of lung metastases

MRI thigh + CT chest

Complete remission → Metastatic disease

b) MLS 8811 t(12;16)

Chromosome 12 (DDIT3)
GCGGGAGGAGAAGGGCCGGCCGCTTGTCGCC
GAAGCAGGAGGACGGCCTGAGAGTAGTGA
CCACGTCTTAGACGGCGCTGAGCAGGGTGT
GCCAAGCGCTCCGCTCGACAAAGAGAGCAC
AGAGCAGACTTCCGCTCACA

MLS 8811 DNA (ng/ct values)

c) Resection of primary tumor → Resection of lung metastasis

MRI thigh + CT chest

Clinical course vs t(12;16) ctDNA

d) Resection of primary tumor → Resection of lung metastasis

MRI thigh + CT chest

Clinical course vs TERT C228T ctDNA

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