Diplomarbeit

New Insights from Long-Term Clinical Use of Circulating Tumor DNA-Based Minimal Residual Disease Monitoring in Translocation-Associated Sarcomas

eingereicht von

Sophie Joch

zur Erlangung des akademischen Grades

Doktorin der gesamten Heilkunde

(Dr.med.univ.)

an der

Medizinischen Universität Graz

ausgeführt an der

Universitätsklinik für Kinder- und Jugendheilkunde / Abteilung für pädiatrische Hämato-Onkologie

in Zusammenarbeit mit der

Universitätsklinik für Orthopädie und Traumatologie

unter der Anleitung der Betreuer

Univ.-Prof. Priv.-Doz. Dr.med.univ. Markus Seidel

Univ.-Prof. Dr.med.univ. Andreas Leithner

Graz, 10.04.2025

Eidesstattliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst habe, andere als die angegebenen Quellen nicht verwendet habe und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Des Weiteren erkläre ich hiermit, dass, sofern bei der Erstellung dieser Arbeit Künstliche Intelligenz (KI) Werkzeuge zur Generierung und/oder Korrektur bestimmter Textpassagen verwendet wurden, dieser Einsatz unter Einhaltung ethischer Grundsätze, akademischer Integrität und den Vorgaben meiner Universität erfolgte, sowie in Folge dies transparent gemacht und in angemessener Weise gekennzeichnet wurde.

Graz, am 10.04.2025 Sophie Joch eh.

Dankesagung

Die Erstellung einer Diplomarbeit, besonders wenn diese eine Veröffentlichung eines Papers beinhaltet, ist nicht selbstverständlich und geht nicht ohne Unterstützung. In diesem Sinne möchte ich mich besonders herzlich bei Markus Seidel bedanken, der mir seit Beginn der Diplomarbeit immer bei Seite gestanden ist und für Fragen jederzeit erreichbar war. Mein Dank gilt auch Prof. Leithner, der mir immer spannenden Input liefern konnte, und Maria Anna Smolle, ohne deren Unterstützung ich bei der Auswertung der Daten nicht weit gekommen wäre. Auch bei Andrea Thüringer möchte ich mich für die großartige Laboreinführung und Unterstützung bedanken. Außerdem gebührt mein Dank all meinen weiteren Coautorinnen und -autoren, Karl Kashofer, Joanna Szkandera, Martin Benesch, Amin El-Heliebi und Bernadette Liegl-Atzwanger, die mir sehr wichtiges Feedback gegeben und mich im Publikationsprozess unterstützt haben.

Auch wenn es oft im Nachhinein so leicht erscheint, ist der Weg bis zu einem abgeschlossenen Medizinstudium immer wieder mit Hürden und Herausforderungen verbunden, bei denen ich jederzeit auf die Unterstützung von meiner Familie und Freunden zurückgreifen konnte. Dankeschön dafür!

Abstract (Deutsch)

Einleitung: Die Nutzung von zirkulierender Tumor-DNA (ctDNA) als Methode zur Überwachung der Krankheitsaktivität bei translokations-assoziierten Sarkomen hat in der klinischen Praxis an Popularität gewonnen. Dennoch gibt es bisher nur wenige Studien zu ihrer klinischen Anwendung. Unsere Studie untersucht die klinische Anwendbarkeit von ctDNA als Biomarker zur Überwachung der minimal residual disease (MRD) bei PatientInnen mit translokations-assoziierten Sarkomen.

Methoden: In dieser retrospektiven Studie korrelierten wir 285 ctDNA-Proben von 34 PatientInnen mit Diagnose eines translokations-assoziierten Sarkoms mit dem klinischen Verlauf und bildgebenden Untersuchungen. Die Blutproben wurden zu mehreren Zeitpunkten während der Nachsorge entnommen (Median: 97 Wochen, Spanne: 7–398).

Ergebnisse: Wir stellten einen signifikanten Zusammenhang zwischen den ctDNA-Werten und dem klinischen Krankheitsverlauf fest, insbesondere bezüglich den Unterschieden der Werte bei PatientInnen in Remission und solchen mit progredienter Erkrankung (p=0,001). In Fällen eines unilokulären Rezidivs (n=3) blieben die ctDNA-Werte nicht nachweisbar, während sie bei PatientInnen mit multilokulären Rezidiven stets erhöht waren (n=14; p=0,008).

Diskussion: Die Überwachung der ctDNA-Werte liefert wertvolle Zusatzinformationen und ermöglicht eine frühzeitige Erkennung von Rezidiven bei PatientInnen mit translokations-assoziierten Sarkomen während der Nachsorge und kann gut in die klinische Praxis integriert werden. Allerdings scheint die alleinige ctDNA-Überwachung nicht ausreichend zu sein, um unilokuläre Rezidive zuverlässig zu erkennen, und sollte durch konventionelle bildgebende Verfahren ergänzt werden.

Abstract (English)

Introduction: Assessment of circulating tumor DNA (ctDNA) as a means to monitor disease activity in translocation-associated tumors has become very popular in clinical practice. However, there are still few studies on its clinical application to date. Our study evaluates the clinical applicability of ctDNA as a biomarker for monitoring minimal residual disease (MRD) in patients with translocation-associated sarcomas.

Methods: In this retrospective study, we correlated 285 ctDNA samples from 34 patients diagnosed with translocation-associated sarcoma with the clinical course and images. Blood samples were collected at multiple time points during follow-up (median: 97 weeks, range: 7–398).

Results: We discovered a significant association between ctDNA levels and the clinical course of the disease, particularly noting differences between patients in remission or with progressive disease ($p = 0.001$). Furthermore, although we noted that ctDNA levels remained undetectable in a few cases of unilocular recurrence ($n = 3$), they were consistently higher in patients with multilocular recurrence ($n = 14$; $p = 0.008$).

Conclusion: Monitoring ctDNA levels provides highly specific, additional information enabling early recurrence detection in patients with translocation-associated sarcomas during the follow-up and can be integrated into clinical practice. However, MRD monitoring by ctDNA quantification alone does not allow the reliable detection of 100% of unilocular recurrences and should be complemented by the use of conventional imaging techniques.

Vorangegangene Veröffentlichungen

An der Medizinischen Universität Graz ist es möglich, eine bereits bei einem S(S)CI gelisteten Journal akzeptierte oder publizierte Arbeit, in der der*die Diplomand*in als Erstautor*in angeführt ist, als Diplomarbeit oder Masterarbeit einzureichen. In diesem Fall wird die veröffentlichte Arbeit 1:1 als Diplomarbeit/Masterarbeit verwendet und auch in dieser Form veröffentlicht.

In diesem Fall liegt die folgende Veröffentlichung mit Einverständniserklärung des Vertrages zur Einreichung als Diplomarbeit an der Medizinischen Universität Graz vor:

Joch S, Smolle MA, Kashofer K, Thüringer A, Szkandera J, Benesch M, El-Heliebi A, Liegl-Atzwanger B, Leithner A, Seidel MG. New Insights from Long-Term Clinical Use of Circulating Tumor DNA-Based Minimal Residual Disease Monitoring in Translocation-Associated Sarcomas. Oncol Res Treat. 2024 Dec 23:1-10. doi: 10.1159/000543223. Epub ahead of print. PMID: 39715595.

Inhalt

Abbildungsverzeichnis

Introduction

Liquid Biopsies

Over the past 10 years, liquid biopsies have emerged as the new superpower in oncology. As the name already suggests, liquid biopsies enable molecular interrogation of different types of cancer using liquid samples of different body fluids. The major advantage of liquid biopsies is that they are minimally invasive and can be used for a variety of purposes, such as prognostic tool, assessing residual disease or early cancer detection and provide a snapshot of a tumor's genetic landscape and its dynamic changes over time (1).

As has been shown in recent years, tumor cells are not only localized in the tumor, but can also detach from the cell cluster, disseminate in the body and are able to extravasate the blood vessels to form metastatic sites (2). These free circulating tumor cells are also called CTCs (Circulating Tumor Cells). In addition to cells, body fluids also contain many other components, including cell free DNA (cfDNA). cfDNA is released into the circulation by cells, in healthy individuals mainly from hematopoietic cells (3). It is composed of short fragments about 135-170 base pairs of DNA and ongoing research suggests that it is not only waste products of cell death but could also have a physiological and pathological function in inflammation and autoimmunity (4). In the case of cancer patients, tumor cells also contribute to the overall pool of cfDNA levels in the blood, in this case called circulating tumor DNA (ctDNA). Its release is related to cell turnover and thus also cell death. The ctDNA concentration depends on various factors. On the one hand, it relies on tumor-intrinsic factors such as tumor burden, tumor proliferation and tumor cell turnover, but also on the tumor entity. For example, patients with colon or pancreatic carcinoma have higher ctDNA levels than patients with kidney carcinoma. On the other hand, the ctDNA level is influenced by tumor-extrinsic factors such as tumor treatment, comorbidities as well as physiological factors such as circadian rhythms (5).

There are several approaches to performing liquid biopsies, depending on the intended use. Various liquids such as cerebrospinal fluid, saliva, nipple aspirate, pleural effusion, ascitic fluid, seminal fluid, cervical fluid, and urine can be analysed, but the most commonly used in cancer diagnostics is blood (6). There are also different

approaches for the isolated sample material, but the most used are the analysis of CTCs and tumor-derived material (circulating tumor DNA (ctDNA), extracellular vehicles (EVs), circulating miRNAs (cfmiRNAs) and TEPs (tumor-bearing platelets)), every single method having its specific applications with advantages and disadvantages. CTCs for example allow structural evaluation of cancer phenotype, offer the use of immunolabeling techniques, and make molecular characterization of the disease possible (7). ctDNA could have a major advantage as a biomarker reflecting the current course of the disease, since its half-life is very short (around 2 hours) (8). Compared to CTCs, ctDNA cannot be cultured, but it is easier to isolate (9).

At the beginning of liquid biopsies, the focus was on single-locus analysis. With increasing development and the more mature technical possibilities such as Whole Genome Sequencing (WGS), it is now possible to track non-genetic factors of cfDNA such as methylation, fragmentation and nucleosome patterns in addition to multiple mutations. These can be used to draw conclusions about specific tissues as well as diseases. In order to process and analyse these large amounts of data, machine learning approaches are increasingly being used (5).

Current clinical applications are the differentiation of unique immune checkpoint blockade response patterns in immunotherapy-based treatments, the predicting response and resistance after targeted therapy, chemotherapy and/or surgery and the evaluation of molecular alterations that are potentially actionable. Most clinical studies have been conducted with colorectal cancers, prostate cancers, breast cancers, melanomas, and lung cancers. The FDA has already approved several kits for liquid biopsies during the last years, for example the FoundationOne Liquid CDx, as the FDA describes a “lab test that detects specific gene mutations found in circulating cell-free DNA (cfDNA) isolated from whole blood plasma specimens, also called liquid biopsy specimens to help doctors identify patients with metastatic castration resistant prostate cancer who may benefit from treatment with the cancer drug RUBRACA [a PARP inhibitor]” (10).

But liquid biopsy also has great potential for broad screening methods for cancer in the general population. The Galleri test developed by the company Grail, a subsidiary of Illumina, promises to screen for more than 50 different types of cancer applying

liquid biopsy methods (11). It is recommended for adults with an elevated risk of cancer such as patients older than 50 years. Nevertheless, the test is not yet an absolute result, as can be read on the website itself, a test result of "no cancer detected" does not completely rule out cancer. The test is also not yet FDA-approved and a randomized control trial is still underway in the UK in order to assess how well the Galleri blood test can help to detect early cancer and therefore reduce the number of late-stage cancers (12).

Thus, liquid biopsies have already found their way into clinical routine for monitoring different types of cancers, especially carcinomas. They will completely change the approaches in oncology, from screening to treatment response control and finding resistance to chemotherapy drugs.

Table 1, Outline of Liquid Biopsies. Adapted from (1)

Liquid Biopsies		
Fluids biopsied	***Examples of Isolated Material***	***Examples of Clinical Applications***
▪ Blood ▪ Urine ▪ Ascites/pleural fluid ▪ Cerebrospinal fluid ▪ Seminal fluid ▪ Nipple aspirate ▪ Saliva ▪ Cervical/vaginal secretions	▪ ctDNA/cfDNA ▪ CTCs ▪ Proteins ▪ DNA ▪ RNA ▪ Methylation ▪ Extracellular Vehicles	▪ Assessment of Minimal Residual Disease (MRD) ▪ Prognostic tool – Predicting therapy response/resistance ▪ Evaluation of Immunotherapy response patterns ▪ Early cancer screening

Liquid Biopsies in Sarcomas

Sarcomas are of particular importance among the malignant mesenchymal diseases. They occur as bone tumors primarily in adolescents and as malignant neoplasms of the soft tissue in small children. Owing to their relatively rare and heterogeneous occurrence and thus the limited capacity to conduct large-scale clinical trials, understanding the molecular mechanisms has become increasingly important in determining the best treatment possible (13). During the last years, there have been several attempts to profile the genomics of multiple sarcomas, for example the Cancer Genome Atlas soft tissue sarcoma (STS) project. Since tumors often release tumor

DNA and proteins into the bloodstream, as mentioned before, examining the plasma for sarcoma-specific changes via liquid biopsies is a promising method for the urgent need of a reliable tumor marker in sarcomas (14). Since there is also DNA from hematopoietic or non-malignant somatic cells in the circulation in addition to tumor DNA, the specific genomics in sarcomas enable to detect samples with tumor-derived material based on their genomic footprint. In contrast to carcinomas, sarcomas have lower incidence and heterogeneity, often resulting in studies of small or mixed cohorts and therefore having difficulties in generating statistical significance.

In this diploma thesis, we focused on liquid biopsies in translocation-associated sarcomas. Based on their stability of chromosomal translocation during progression of disease and clonal homogeneity, their genomic footprint should be even a more reliable source as a molecular DNA marker compared to tumors with a high mutation rate in disease progression and thus are an ideal candidate for liquid biopsies (14).

Considering the current literature, there is no report or guideline for clinical application of liquid biopsy in translocation-associated tumors. The previously published article of Seidel et al. (15) reported about six preselected cases of patients with Ewing sarcomas and their follow-up in a clinical setting with liquid biopsies. The aim of this diploma thesis was to broaden our patient population and include all patients with translocation-associated tumors in our study, analyse their follow-up with liquid biopsies and therefore correlate the patient-specific biomarker with their clinical course. The aim was to include more patients and to have a longer period of follow-up than in the previous study.

Works Cited

1. Nikanjam M, Kato S, Kurzrock R. Liquid biopsy: current technology and clinical applications. J Hematol OncolJ Hematol Oncol. 2022 Sep 12;15(1):131.

2. Cheng X, Cheng K. Visualizing cancer extravasation: from mechanistic studies to drug development. Cancer Metastasis Rev. 2021 Mar;40(1):71–88.

3. Heitzer E, Auinger L, Speicher MR. Cell-Free DNA and Apoptosis: How Dead Cells Inform About the Living. Trends Mol Med. 2020 May 1;26(5):519–28.

4. Han DSC, Lo YMD. The Nexus of cfDNA and Nuclease Biology. Trends Genet TIG. 2021 Aug;37(8):758–70.

5. Moser T, Kühberger S, Lazzeri I, Vlachos G, Heitzer E. Bridging biological cfDNA features and machine learning approaches. Trends Genet. 2023 Apr 1;39(4):285–307.

6. Roy D, Lucci A, Ignatiadis M, Jeffrey SS. Cell-free circulating tumor DNA profiling in cancer management. Trends Mol Med. 2021 Oct 1;27(10):1014–5.

7. Adashek JJ, Janku F, Kurzrock R. Signed in Blood: Circulating Tumor DNA in Cancer Diagnosis, Treatment and Screening. Cancers. 2021 Jul 18;13(14):3600.

8. Rostami A, Lambie M, Yu CW, Stambolic V, Waldron JN, Bratman SV. Senescence, Necrosis, and Apoptosis Govern Circulating Cell-free DNA Release Kinetics. Cell Rep [Internet]. 2020 Jun 30 [cited 2023 Oct 24];31(13). Available from: https://www.cell.com/cell-reports/abstract/S2211-1247(20)30811-1

9. Abbou SD, Shulman DS, DuBois SG, Crompton BD. Assessment of circulating tumor DNA in pediatric solid tumors: The promise of liquid biopsies. Pediatr Blood Cancer. 2019 May;66(5):e27595.

10. fda.gov [Internet]. [cited 2023 Jul 3]. FoundationOne Liquid CDx – P190032. Available from: https://www.fda.gov/medical-devices/recently-approved-devices/foundationone-liquid-cdx-p190032

11. Galleri® Test [Internet]. [cited 2023 Oct 24]. About Galleri. Available from: https://www.galleri.com/what-is-galleri

12. Neal RD, Johnson P, Clarke CA, Hamilton SA, Zhang N, Kumar H, et al. Cell-Free DNA–Based Multi-Cancer Early Detection Test in an Asymptomatic Screening Population (NHS-Galleri): Design of a Pragmatic, Prospective Randomised Controlled Trial. Cancers. 2022 Jan;14(19):4818.

13. Burns J, Brown JM, Jones KB, Huang PH. The Cancer Genome Atlas. Surg Oncol Clin N Am. 2022 Jul;31(3):559–68.

14. Krumbholz M, Hellberg J, Steif B, Bäuerle T, Gillmann C, Fritscher T, et al. Genomic *EWSR1* Fusion Sequence as Highly Sensitive and Dynamic Plasma Tumor Marker in Ewing Sarcoma. Clin Cancer Res. 2016 Sep 1;22(17):4356–65.

15. Seidel MG, Kashofer K, Moser T, Thueringer A, Liegl-Atzwanger B, Leithner A, et al. Clinical implementation of plasma cell-free circulating tumor DNA quantification by digital droplet PCR for the monitoring of Ewing sarcoma in children and adolescents. Front Pediatr. 2022 Aug 15;10:926405.

Oncology Research and Treatment

Research Article

Oncol Res Treat
DOI: 10.1159/000543223

Received: August 30, 2024
Accepted: December 9, 2024
Published online: December 23, 2024

New Insights from Long-Term Clinical Use of Circulating Tumor DNA-Based Minimal Residual Disease Monitoring in Translocation-Associated Sarcomas

Sophie Joch[a] Maria Anna Smolle[b] Karl Kashofer[c] Andrea Thüringer[c] Joanna Szkandera[d] Martin Benesch[a] Amin El-Heliebi[e,f] Bernadette Liegl-Atzwanger[c] Andreas Leithner[b] Markus G. Seidel[a,g]

[a]Division of Paediatric Haematology-Oncology, Department of Paediatrics and Adolescent Medicine, Medical University of Graz, Graz, Austria; [b]Department of Orthopaedics and Trauma, Medical University of Graz, Graz, Austria; [c]Institute of Pathology, Medical University of Graz, Graz, Austria; [d]Division of Oncology, Department of Internal Medicine, Medical University of Graz, Graz, Austria; [e]Division of Cell Biology, Histology and Embryology, Gottfried Schatz Research Center, Medical University of Graz, Graz, Austria; [f]European Liquid Biopsy Society (ELBS), Hamburg, Germany; [g]Styrian Children's Cancer Research Unit for Cancer and Inborn Errors of the Blood and Immunity in Children, Medical University of Graz, Graz, Austria

Keywords
Liquid biopsy · Translocation-associated sarcomas · Cell-free circulating tumor DNA · Minimal residual disease monitoring

Abstract

Introduction: Assessment of circulating tumor DNA (ctDNA) as a means to monitor disease activity in translocation-associated tumors has become very popular in clinical practice. However, there are still few studies on its clinical application to date. Our study evaluates the clinical applicability of ctDNA as a biomarker for monitoring minimal residual disease (MRD) in patients with translocation-associated sarcomas. ***Methods:*** In this retrospective study, we correlated 285 ctDNA samples from 34 patients diagnosed with translocation-associated sarcoma with the clinical course and images. Blood samples were collected at multiple time points during follow-up (median: 97 weeks, range: 7–398). ***Results:*** We discovered a significant association between ctDNA levels and the clinical course of the disease, particularly noting differences between patients in remission or with progressive disease (p = 0.001). Furthermore, although we noted that ctDNA levels remained undetectable in a few cases of unilocular recurrence (n = 3), they were consistently higher in patients with multilocular recurrence (n = 14; p = 0.008). ***Conclusion:*** Monitoring ctDNA levels provides highly specific, additional information enabling early recurrence detection in patients with translocation-associated sarcomas during the follow-up and can be integrated into clinical practice. However, MRD monitoring by ctDNA quantification alone does not allow the reliable detection of 100% of unilocular recurrences and should be complemented by the use of conventional imaging techniques.

Plain Language Summary

This study investigates the emerging use of circulating tumor DNA (ctDNA) as a method to monitor the tumor burden in patients with a specific group of cancers called

Andreas Leithner and Markus G. Seidel shared last authorship.

karger@karger.com
www.karger.com/ort

Correspondence to:
Maria Anna Smolle, maria.smolle@medunigraz.at
Karl Kashofer, karl.kashofer@medunigraz.at

translocation-associated sarcomas. These rare cancers result from particular genetic changes called translocations. ctDNA is genetic material shed into the blood by tumor cells. Thus, ctDNA (with the tumor-specific translocation) detected in the blood of patients may represent a surrogate parameter for the presence of active tumor cells, serving as a genetic tumor marker. We analyzed 285 blood samples from 34 patients with translocation-associated sarcomas taken at different time points in the patients' follow-up period of 7–398 weeks (2 years on average) to see how ctDNA levels correlated with the course of their disease. Our study found that ctDNA levels were strongly linked to a treatment response or recurrence of the disease. In tumor-free patients, ctDNA levels were undetectable. However, where the disease returned – especially if these levels were detectable in multiple areas – ctDNA levels went up again. In summary, using ctDNA analyses in translocation-associated cancers is a valuable, rather sensitive, and highly specific additional tool for monitoring the treatment response and for early detection of a recurring disease.

Introduction

Sarcomas are of particular importance among pediatric neoplasms due to their frequency, severity, and distinct age-related patterns. These primarily occur as bone tumors in adolescents and as soft tissue sarcomas in young children. Their relatively rare occurrence and heterogeneous presentation limits the possibility to conduct large-scale clinical trials. Therefore, understanding molecular mechanisms in sarcomas, such as gene fusions, mutations, epigenetic modifications, pathway dysregulation, and tumor microenvironment interactions, have become increasingly important for determining the most appropriate, personalized cancer treatment [1]. As sarcomas release tumor DNA and RNA into the bloodstream, liquid biopsies enable the examination of plasma for sarcoma-specific cell products; therefore, they address the urgent need for a reliable surveillance method in patients with sarcomas. Additionally, the genomic breakpoint of a translocation presents a unique DNA feature that represents an excellent target for tumor-specific, highly sensitive, quantitative PCR assays. Consequently, translocation-associated sarcomas are ideal candidates for liquid biopsies [2]. Kjær et al. [3] have demonstrated that the detection of circulating tumor DNA (ctDNA) is the most practical method in clinical practice for the diagnosis, prognosis prediction, and disease monitoring of sarcomas. The best evidence for ctDNA detection in sarcomas currently exists for Ewing sarcomas, alveolar rhabdomyosarcomas, and synovial sarcomas. Changes in disease burden are reflected by the ctDNA levels [4–6], and in Ewing sarcoma, performing a liquid biopsy not only enables the examination of a measurable parameter for early risk stratification [7, 8] but enables correlations to be drawn with metabolic tumor parameters [9]. In comparison to the three sub-entities of translocation-associated sarcomas described above, few scientific publications exist on liquid biopsies of Ewing-like sarcomas, myxoid liposarcomas, or desmoplastic small round cell tumors (DSRCTs). In the case of Ewing-like sarcoma and DSRCTs, this is primarily due to their rarity. However, two studies have shown that liquid biopsies are also feasible in less common sarcoma sub-entities [10, 11].

In general, liquid biopsies for monitoring minimal residual disease are becoming more popular in the clinical routine, but no guidelines that describe cut-offs or reports on the clinical application of liquid biopsy in translocation-associated tumors have been written. Seidel et al. [12] reported on preselected cases of patients with Ewing sarcoma and their follow-up in a clinical setting, investigating in the feasibility of monitoring ctDNA levels and correlating them with the course of the disease. The authors demonstrated that a clinical follow-up with liquid biopsy is feasible, but only a few patients with Ewing sarcoma were included in the study [12].

To address this limitation, we broadened our patient population and included patients with other subtypes of translocation-associated sarcomas in this study. Our hypothesis was that ctDNA levels correlated with the patients' clinical courses; therefore, these levels constituted a reliable and auxiliary marker for clinical decision-making.

Methods

Study Design and Population

This observational cohort study retrospectively included 34 patients (aged 2–78 years at initial diagnosis) with translocation-associated sarcomas who were treated at a single university hospital and had undergone liquid biopsy analysis using a previously described method [12]. The main inclusion criterion was the pathologic diagnosis of translocation-associated sarcoma as confirmed by RNA sequencing (see online suppl. Table 1; for all online suppl. material, see https://doi.org/10.1159/000543223). All possible translocation-associated tumor entities with chromosomal translocations considered for the study are also listed in online supplementary Table 1, of which only 6 were represented in our clinic. Liquid biopsy samples containing ctDNA were obtained between January 2016 and March 2024. No age restrictions or specific treatment history were applied for inclusion. Owing to the methodology employed, 5 patients were excluded, as an adequate breakpoint mutation in their primary tumor sample could not be identified due to the lack of a detectable gene fusion or the unavailability of primary tumor

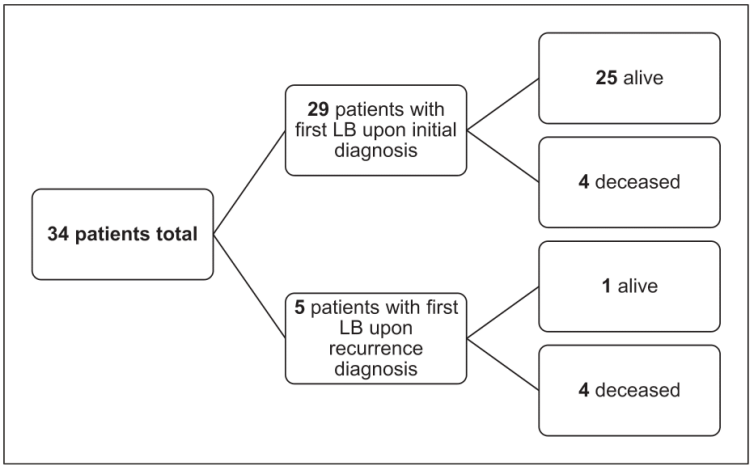

Fig. 1. Flowchart of included patients. LB, liquid biopsy.

material. Of all patients investigated, 29 had their first liquid biopsy taken at primary tumor diagnosis (15 patients) or during follow-up (14 patients), and 5 upon the recurrence of their disease (Fig. 1). After the implementation of ctDNA liquid biopsy monitoring in translocation-positive sarcomas into the clinical routine at our institution [12], the time points triggering a ctDNA analysis were chosen at the physicians' discretion. These were often chosen to monitor the treatment response or minimal residual disease (e.g., during neoadjuvant or adjuvant chemotherapy) or to obtain an auxiliary tumor marker at time points coinciding with those of imaging studies during long-term follow-up. During an event, 110 of the total 285 ctDNA samples were taken (online suppl. Table 2). Due to the retrospective nature of the study, no diagnostic study plan predefined the time points at which ctDNA analyses had to be consecutively undertaken. The irregular intervals at which ctDNA investigations were performed in our cohort precluded a detailed study of the ctDNA kinetics in response to therapy or the "lead-in" time before a clinical diagnosis of recurrence. Follow-up was conducted for each patient from diagnosis until end of March 2024 or until the patient's death. The study was approved by the Institutional Review Board and the Ethics Committee of the Medical University of Graz (IRB00002556; 28397ex15/16) and performed under the current Good Clinical Practice guidelines by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH).

Data Collection

Blood samples were collected according to the established in-house-practice with PAXgene Blood ccfDNA Tubes® (PreAnalytiX, Hombrechtikon, Switzerland) and as previously described [12]. Samples were obtained from patients with translocation-associated sarcomas either upon initial diagnosis or upon recurrence (i.e., in patients already in follow-up who had been treated for their sarcomas without liquid biopsies upon their initial diagnosis), as well as at dedicated time points during treatment (i.e., chemotherapy, radiotherapy) and follow-up. The timing of follow-ups was chosen based on the tumor entities and risk stratification with reference to international guidelines and/or in-house standards of care, as tailored to each patient. Tumor tissue from a biopsy or from resection specimens was used, depending on whether the patient was treatment-naive or had already been treated with chemotherapy or radiotherapy before surgery. Tumor-/patient-specific breakpoint mapping was performed as described previously [12]. In brief, we performed targeted RNA-Seq with next-generation sequencing by using the Archer fusion plex sarcoma panel (ARCHERDX, Invitae, Boulder, CO, United States) in tumor tissue to identify the translocation (online suppl. Table 1). We then performed whole genome sequencing to detect the exact individual breakpoint of the fusion partners and designed primers for quantitative fusion gene detection in blood plasma [12]. Primer sequences may be provided by the corresponding authors upon legitimate request. ctDNA was quantified using a specific digital droplet polymerase chain reaction (ddPCR) designed based on the translocation breakpoint. The original tumor DNA was used as a positive control in all ddPCR assays. All samples were either directly analyzed or stored at −80°C until further processing and analysis. All ddPCRs were carried out according to a reported protocol [12] and the results were reported by specialized pathologists at the university's pathology institute.

Definitions and Classifications

In addition to using a liquid biopsy method, the course of the disease was also tracked clinically and by applying imaging techniques. To track the clinical course of the disease, we differentiated between the stages of stable disease, progression, regression, and no detectable tumor. For each individual ctDNA value, we analyzed changes in the clinical course and images within a period of 2 weeks before and after the ctDNA sampling and matched these to the corresponding ctDNA value.

Recurrence was differentiated into unilocular and multilocular recurrence. Unilocular recurrences were defined as the tumor recurring in a single location. Multilocular recurrence refers to any case in which multiple sites of tumor recurrence were identified, regardless of whether these occurred at different body locations (e.g., lungs and soft tissues) or several sites within a single organ (e.g., multiple lung metastases).

Statistical Analysis

Continuous variables are presented as medians with ranges. Numbers are given with valid percentages. Differences in ctDNA amounts in liquid biopsy samples (copies/mL) between groups were compared by applying the Mann-Whitney U test and the Kruskal-Wallis tests, as appropriate. For these analyses, each clinical event and liquid biopsy measurement was counted separately. A p value of < 0.05 was considered statistically significant. All analyses were carried out with the Stata software package (Version 16.1, StataCorp, College Station, TX, USA).

Results

Patient Characteristics – First Liquid Biopsy at Primary Tumor Diagnosis or during Follow-Up

Of the 29 patients whose first liquid biopsy was taken upon primary tumor diagnosis (15 patients) or during the follow-up (14 patients), 13 patients were female (44.8%) and 16 were male (55.2%); the median age at diagnosis was 16 years (range, 2–78 years). Most patients had been diagnosed with Ewing sarcoma (15/29; 51.7%). At the time of diagnosis, 12 patients presented with metastatic disease (41.4%), and 17 with localized disease (58.6%). Twenty-two patients underwent chemotherapy (75.8%), and 20 underwent surgery of the primary tumor (71.4%). In addition, 53.6%, 7.1%, and 14.2% of patients received conventional radiotherapy, proton therapy, or both, respectively. A median of 6 liquid biopsy samples had been obtained per patient (range, 1–36). The median follow-up was 97 weeks (range, 7–398 weeks). During the follow-up, recurrence occurred in 7 patients (25%). Detailed characteristics together with tumor locations are outlined in Table 1.

Patient Characteristics – First Liquid Biopsy upon Recurrence

Of the 5 patients whose first liquid biopsy was taken upon recurrence, three were female, and the median age was 13 (range, 11–19) years. Three of these 5 patients had Ewing sarcoma and two had alveolar rhabdomyosarcoma (Table 1).

Correlation of Liquid Biopsy Levels with Disease Burden

The median liquid biopsy ctDNA levels (copies/mL) were 307.9 (range, 0–2,648.1) in patients whose first measurement was obtained upon initial tumor diagnosis ($n = 15$), 0 (range, 0–1,757.1) in patients whose first liquid biopsy was obtained during the follow-up ($n = 14$), and 0 (range, 0–15.9) in patients whose first liquid biopsy was taken upon recurrence ($n = 5$).

ctDNA levels associated with course of disease at respective time points are shown in Figure 2. The median copies/mL were significantly lower in patients with tumor regression or in remission (0 [range, 0–58.7 copies/mL]) than in patients with progressive disease (14.5 [range, 0–4,203.7 copies/mL]) during the follow-up ($p = 0.001$; Fig. 2a). Similarly, liquid biopsy levels correlated with clinical tumor dynamics: Those patients that had low levels were in regression or remission (0 [range, 0–58.7 copies/mL]) or in a stable disease state (0 [range, 0–1,757.1 copies/mL]), while those with high levels were in a progressive disease state (15.2 [range, 0–4,203.7]; $p = 0.001$; Fig. 2b). No significant correlation was found between the tumor volume (assessed via imaging) and the numbers of breakpoint copies in the liquid biopsy samples ($\rho = 0.251$; $p = 0.331$).

Lead-In Time

The lead-in time, defined as the interval between an increase in ctDNA levels and the clinical diagnosis of a recurrence, was calculated for patients with a multilocular recurrence preceded by consistently undetectable ctDNA levels (0 copies/mL). Among the four (4) patients meeting these criteria, the median lead-in time was six (6) days prior to the clinical diagnosis, with a range of 0–20 days.

Differences between Unilocular/Multilocular Recurrence

When only considering the liquid biopsy sample taken at time of recurrence (17 recurrence events in 8 patients), median copies/mL were lower in case of unilocular recurrence (3 events; 0 [range, 0–0]) compared to those with multilocular recurrence (14 events; 42.2 [13.3–163.0]; $p = 0.008$).

Preselected Cases with Special Clinical Significance

The overall course of ctDNA level variation as measured for each patient is shown in Figure 3. The following patients highlight the potential of ctDNA detection as a valuable tool for monitoring therapy response, early recurrence detection, and managing rare sarcomas.

Table 1. Detailed characteristics of patients, separated by first liquid biopsy taken upon diagnosis (n = 30), and first liquid biopsy taken upon recurrence (n = 4)

	Primary diagnosis, n = 29	Recurrence, n = 5
Age at diagnosis (in years; median, range)	16 [2–78]	13 [11–19]
Female sex	13/29 (44.8%)	3/5 (60.0%)
Histology		
Ewing sarcoma	15/29 (51.7%)	3/5 (60.0%)
Synovial sarcoma	7/29 (24.1%)	0/5 (0.0%)
Alveolar rhabdomyosarcoma	2/29 (6.9%)	2/5 (40.0%)
Myxoid liposarcoma	2/29 (6.9%)	0/5 (0.0%)
DSRCT	1/29 (3.4%)	0/5 (0.0%)
CIC-DUX4 sarcoma	1/29 (3.4%)	0/5 (0.0%)
EWSR1-non-ETS fusion sarcoma	1/29 (3.4%)	0/5 (0.0%)
Localized disease	17/29 (58.6%)	3/5 (60%)
Surgery	20/28[a] (71.4%)	5/5 (100%)
Chemotherapy	22/29 (75.8%)	5/5 (100%)
Radiotherapy		
Conventional radiotherapy	15/28[a] (53.6%)	4/5 (80%)
Proton beam therapy	2/28[a] (7.1%)	1/5 (20%)
Conventional radiotherapy + proton beam therapy	4/28[a] (14.2%)	0/5 (0%)
Tumor location		
Soft tissues		
Thigh	6/29 (20.7%)	1/5 (20.0%)
Forearm	3/29 (10.3%)	0/5 (0.0%)
Lower leg	1/29 (3.4%)	0/5 (0.0%)
Planta pedis	1/29 (3.4%)	0/5 (0.0%)
Paravertebral	1/29 (3.4%)	0/5 (0.0%)
Kidney	1/29 (3.4%)	0/5 (0.0%)
Peritoneum	1/29 (3.4%)	0/5 (0.0%)
Bone		
Femur	4/29 (13.8%)	0/5 (0.0%)
Pelvis	4/29 (13.8%)	0/5 (0.0%)
Ribs	3/29 (10.3%)	2/5 (40.0%)
Spine	1/29 (3.4%)	0/5 (0.0%)
Tibia	1/29 (3.4%)	1/4 (25.0%)
Skull	0/29 (0.0%)	1/4 (25.0%)
Liquid biopsies per patient (median, range)	6 (1–36)	8 (6–15)
Recurrence during follow-up	7/28[a] (25%)	N/A
Status at last follow-up		
Alive	25/29 (86.2%)	1/5 (20%)
Deceased	4/29 (13.8%)	4/5 (80%)
Median follow-up (in weeks; median, range)	97 [7–398]	195 [147–750]

DSRCT, desmoplastic small round cell tumor. [a]One patient lost to follow-up early during treatment phase.

Early Recurrence Detection after Complete Remission

In 3 patients (IDs 12, 22, and 27) ctDNA levels were instrumental for detecting early stage recurrences of Ewing sarcoma. Each patient had initially reached complete remission and ctDNA levels at 0 copy/mL following neoadjuvant and adjuvant treatment. Subsequent increases in ctDNA levels could be detected before these recurrences could be detected by imaging. Whole-body imaging, performed promptly upon detecting elevated ctDNA levels, confirmed new metastases in all 3 of these patients.

ctDNA as a Supportive Parameter in Case of Unclear Imaging Results

A 16-year-old male patient (ID 20) was diagnosed with Ewing sarcoma of the 3rd to 6th rib (total tumor volume of approximately 980 mL), expanding to the mediastinal

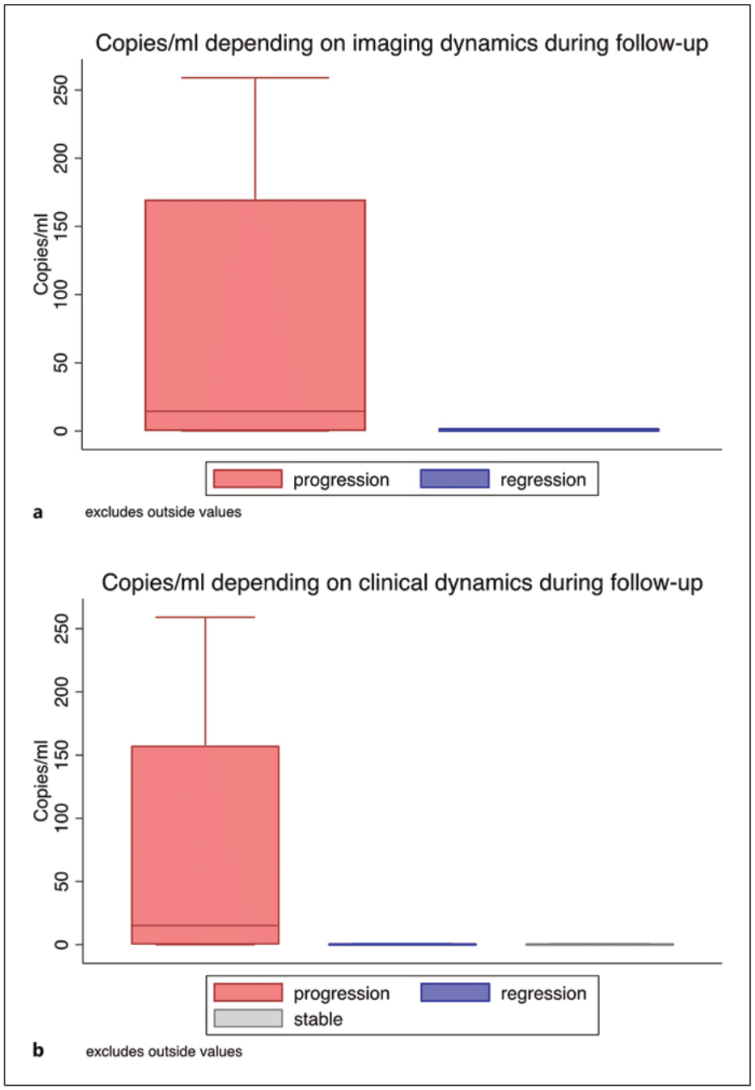

Fig. 2. ctDNA levels in liquid biopsy samples (copies/mL) depending on course of disease on imaging (**a**) and clinical follow-up (**b**).

and lateral pleura as well as to the aortic arch. The patient underwent surgery with clear margins but underwent a prolonged postoperative recovery period, as well as neoadjuvant and adjuvant chemotherapy (EWING 2008 protocol). Sixty-seven weeks after his initial diagnosis and 15 weeks after the last adjuvant chemotherapy cycle, a PET-CT showed a newly occurring left pleural soft tissue lesion indicative of secondary metastasis.

At the time of the primary diagnosis, the patient showed a positive response to chemotherapy, as indicated by a decrease in ctDNA levels from 501.7 copies/mL to 1.7 copies/mL within 1 month of starting therapy (Fig. 4). After the ninth cycle of neoadjuvant chemotherapy, ctDNA levels became undetectable (0 copies/mL) and remained at this level. In the weeks following a suspected recurrence, the liquid biopsy results continued to show no detectable ctDNA. After two CT-guided biopsies which were incompatible with a recurrence of EWS, two open biopsies were performed, ultimately enabling the diagnosis of benign extra-abdominal fibromatosis in the vicinity of the original surgical field.

Liquid Biopsy in Patients with Rare Sarcomas

We also included 3 patients with rare sub-entities of translocation-associated sarcomas, where the application of liquid biopsies is not commonly reported. The first patient (ID 3) was an 18-year-old male patient diagnosed with DSRCT localized in the abdominal-peritoneal region. Despite

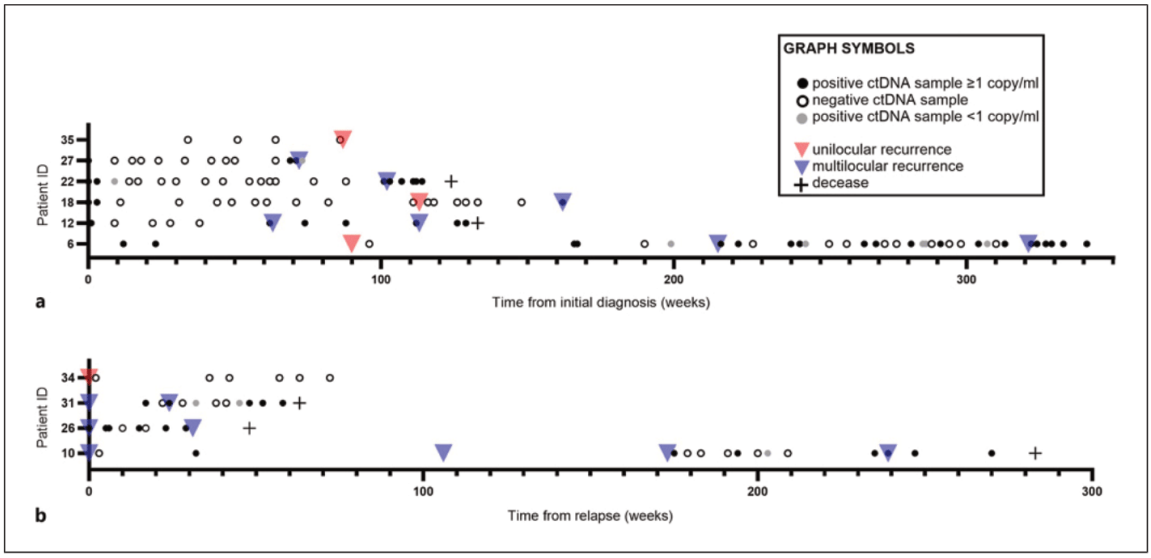

Fig. 3. ctDNA levels in samples of patients with recurrence and ctDNA levels in samples within 2 weeks before or after the recurrence. Each point represents one sample used for ctDNA detection. **a** All patients subjected to liquid biopsies since their initial sarcoma diagnosis. **b** All patients subjected to liquid biopsies since recurrence diagnosis.

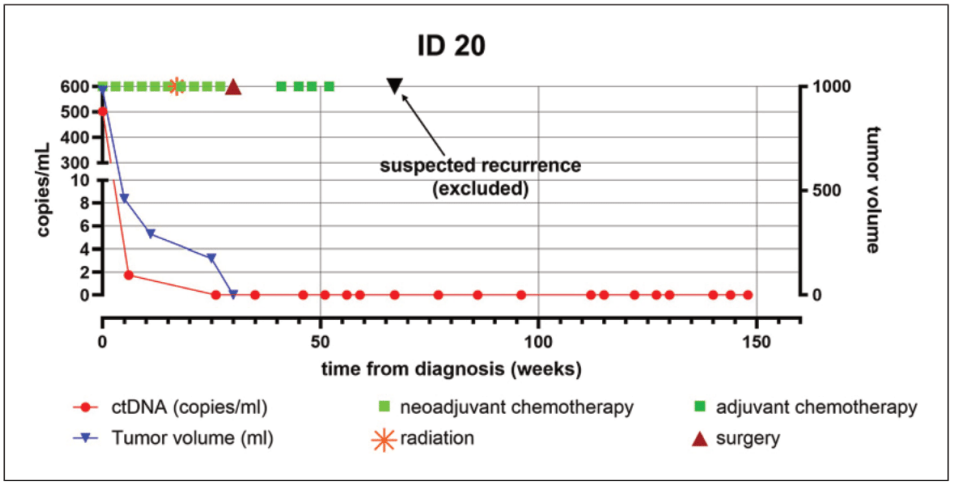

Fig. 4. Course of disease of patient ID 20.

the presence of significant nodular confluent tumor masses in the lower abdomen and small pelvis, all ctDNA samples taken during the follow-up (the first one was taken 3 weeks post-diagnosis) remained negative. We conclude that this specific tumor did not release DNA into the circulation.

The second patient, a 10-year-old boy (ID 25), initially suspected to have a juvenile bone cyst in the femur, underwent surgery. A molecular pathological examination subsequently identified an EWSR1-non-ETS fusion (NFATc2) sarcoma. As no sarcoma had been initially suspected, no ctDNA samples had been taken before surgery; however, after diagnosis had been reached, ctDNA liquid biopsy samples were obtained during follow-up as a monitoring marker. All samples drawn ($n = 4$) up to the last follow-up at the time point of 135 weeks remained negative for ctDNA, correlating with negative imaging results.

The third patient, a 12-year-old girl (ID 45), was diagnosed with a *CIC::DUX4* fusion-positive sarcoma in the tibialis anterior muscle and multiple pulmonary metastases. The initial test for ctDNA, conducted after the first cycle of neoadjuvant chemotherapy, was positive (10.7 copies/mL). Currently, the patient is still under therapy, with latest liquid biopsy result (13 weeks after primary diagnosis) indicating that the ctDNA levels have already declined to 0 copies/mL.

Discussion

Our study revealed that ctDNA levels correlate with the clinical course in patients with sarcoma and that these levels constitute a reliable, specific auxiliary marker that supports clinical decision-making, allowing its application in clinical routine. However, ctDNA monitoring alone appears insufficient to detect unilocular recurrence, and should be complemented by the use of conventional imaging techniques. Given that we investigated the clinical applicability of ctDNA detection, we will focus on this analyte in the remainder of this section.

As expected, based on the results of previously published studies by Krumbholz et al. [2], Kjær et al. [3], and Tombolan et al. [5], we show that ctDNA levels in patients with known sarcomas correlate closely with clinical progression. Specifically, a significant difference in ctDNA levels was found between patients who responded to therapy, were in partial or complete remission, and who had a progressive form of the disease. Due to this difference, we consider ctDNA detection to be an important minimally invasive method that can be used to perform longitudinal monitoring in oncological aftercare, as complemented by imaging procedures. A reduction in ctDNA levels has been shown to correlate closely with a response to therapy, as noted by Krumbholz et al. [7]. The detection of increasing ctDNA levels after a prolonged period of non-detectability is a strong indication of recurrence, warranting the initiation of imaging procedures as soon as possible.

Although the ctDNA levels correlated closely with the clinical course in most cases, some notable exceptions were observed. In our study, the ctDNA levels consistently remained at 0 copies/mL in all patients who experienced unilocular recurrence (three events of unilocular recurrence), despite the clinical evidence of disease progression. This contrasts with the pattern of ctDNA levels seen in cases of multilocular recurrence, where an increase in the ctDNA levels was consistently observed (14 events; $p = 0.008$). Therefore, using a liquid biopsy method in combination with ddPCR (to detect patient-specific translocation breakpoints) cannot replace imaging as the sole method for longitudinal monitoring. Stegmaier et al. [13] discovered that patients with primary localized alveolar rhabdomyosarcomas do not present with a positive liquid biopsy. However, these authors used cell-free exosomal RNA as an analyte, not ctDNA. This finding, while not directly applicable to unilocular recurrence, suggests limitations in the use of ddPCR to detect patient-specific fusion breakpoints over the course of the disease. The absence of ctDNA in the case of unilocular recurrence has not been explicitly described yet. There are two explanations for the absence of detectable ctDNA in unilocular recurrences: (1) The ctDNA shed by a single metastatic lesion is below the threshold of detection, and (2) biological differences in the tumor microenvironment or ctDNA release mechanisms could play a role [14]. In general, it is important to note that the tumor localization might affect detectable ctDNA levels, but factors such as the amount of necrotic tumor material, the time since the last therapy, or daily fluctuations could also play a significant role in ctDNA release. Further investigations into sarcoma metabolism and its influence on ctDNA levels would be highly beneficial, as these would enable researchers to develop complementary diagnostic tools that can be used to more accurately detect the presence of localized metastases. However, Bodlak et al. [15] showed that ctDNA detection is superior to circulating tumor ribonucleic acids (ctRNA) detection, at least in Ewing sarcoma. Unlike Abbou et al. [11], we did not detect ctDNA in a patient with DSRCT, despite the massive extent of this patient's tumor [11], and, to date, a biological or technical explanation for this observation is lacking. Because factors such as the quality of plasma samples and the establishment of suitable primer-probe assays can affect ctDNA detection, the primers used for ddPCR in our study were consistently tested using the initially positive tumor tissue sample from each patient. Given the rarity of this tumor entity, more research is needed to evaluate the clinical use of this detection method.

The heterogeneity of our already small patient cohort is a limitation of this study. As Abbou et al. [16] mentioned, a multi-institutional effort is necessary to conduct sufficiently large studies. In this context, the results of our study do not uniformly support the detection of ctDNA as a valuable screening tool for every translocation-associated sarcoma sub-entity. Another limitation is the fact that ctDNA sampling took place at varying time intervals and events, both initially and during the follow-up. Although liquid biopsies had already been implemented into our clinical routine, the first liquid biopsies were not always obtained upon initial diagnosis due to secondary patient referral, but during the treatment or follow-up period. Standardizing intervals and time points for ctDNA sampling would have allowed for a more precise evaluation of the time-to-non-detectability and the impact of chemotherapy on ctDNA levels, as well as for an assessing of a potential lead-in period, when ctDNA may be detected before clinical signs manifest.

Our ctDNA analysis approach does not offer differential diagnostic capabilities compared to methods like structural variant detection [13], and it requires prior knowledge of the pathologic tumor subtype. Low-pass whole genome sequencing could broaden the applicability of ctDNA analyses to a wider patient population in the future [17, 18].

Our study included more patients with diverse sarcoma sub-entities in the clinical setting than Seidel et al. [12]: we collected a total of 285 liquid biopsy samples for ctDNA analysis and correlated the ctDNA levels with the clinical course of each patient. By taking 6 to 8 samples per patient, we exceeded the number of samples taken per patient in a previous study on this topic [7]. Moreover, our present study had a longer follow-up period than Seidel et al. [12] and included ultra-rare soft tissue sarcoma subtypes as well.

Compared to the method of ctDNA analysis in gastrointestinal stromal tumors, ctDNA analysis in translocation-associated sarcomas is not as advanced. The former method has proven effective for identifying both known and novel mutations that might not be detected with traditional tissue DNA testing [19]. This means that it is particularly useful for assessing the risk of disease progression and monitoring responses to therapy. Further research on the application of ctDNA detection in translocation-associated sarcomas could potentially achieve similar advancements.

In conclusion, we demonstrated that monitoring ctDNA levels in patients with translocation-associated sarcomas is a feasible method for applying in the clinical routine and serves as an early indicator of recurrence. Nevertheless, ctDNA monitoring alone might not be suitable for the detection of unilocular recurrence; therefore, this method should still be accompanied by conventional imaging.

Acknowledgment

We sincerely thank Ellen Heitzer for her important contributions in the field of liquid biopsies and especially to the baseline pilot study, which made it possible to implement ctDNA quantification as method for detecting residual disease into the clinical routine at our institution. The authors thank Sara Crockett (www.saras-science.com) for scientific language editing.

Statement of Ethics

This study protocol was reviewed and approved by the Ethics Commission, Medical University of Graz, Approval No. IRB00002556; 28397ex15/16. A written informed consent to participate in this study was provided by the participants and, in case of underaged participants, by their legal guardian/next of kin according to the Ethics Committee approval.

Conflict of Interest Statement

S.J., K.K., B.L.-A., and A.T. have no conflicts of interest to declare. M.A.S. has received travel support from ImplanTec, Alphamed, implantcast, and PharmaMar outside of the submitted work. J.S. has received sponsorship and research funds from Eisai, PharmaMar, and Roche and has received payment or other (financial) remuneration from Bayer, Amgen, PharmaMar, and Roche. M.G.S. has received research funding from Amgen and Takeda (paid to institution), consultancy honorary from Pharming, Amgen, and Novartis, and a conference travel grant from CSL Behring. A.E. has received research funding from Qiagen (paid to institution) and received remuneration from Illumina. A.L. reports receiving institutional educational grants by Alphamed, Medacta, and Johnson & Johnson. The funders had no role in the design of this study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

Funding Sources

M.G.S.'s. research unit was in part funded by the Steirische Kinderkrebshilfe [Styrian Children's Cancer Aid].

Author Contributions

S.J., J.S., M.B., A.L., and M.G.S. collected patient clinical data; A.T., K.K., and B.L.-A. analyzed patient tumor and plasma samples; A.L., M.G.S., and A.E.-H. designed the study; S.J. and M.A.S. analyzed the data, prepared the tables and figures, and drafted the manuscript, and all authors edited the final manuscript.

Data Availability Statement

All data that were obtained in the present study are shown in the figures, tables, or text of the manuscript. If additional information or a different data format is required, these are available from the corresponding author upon reasonable request.

References

1 Grünewald TGP, Cidre-Aranaz F, Surdez D, Tomazou EM, De Álava E, Kovar H, et al. Ewing sarcoma. Nat Rev Dis Primer. 2018;4(1):5. https://doi.org/10.1038/s41572-018-0003-x

2 Krumbholz M, Hellberg J, Steif B, Bäuerle T, Gillmann C, Fritscher T, et al. Genomic *EWSR1* fusion sequence as highly sensitive and dynamic plasma tumor marker in ewing sarcoma. Clin Cancer Res. 2016;22(17):4356–65. https://doi.org/10.1158/1078-0432.CCR-15-3028

3 Kjær EKR, Vase CB, Rossing M, Ahlborn LB, Hjalgrim LL. Detection of circulating tumor-derived material in peripheral blood of pediatric sarcoma patients: a systematic review. Transl Oncol. 2023;34:101690. https://doi.org/10.1016/j.tranon.2023.101690

4 Anderson P, Ghisoli M, Crompton BD, Klega KS, Wexler LH, Slotkin EK, et al. Pilot study of recurrent ewing's sarcoma management with vigil/temozolomide/irinotecan and assessment of circulating tumor (ct) DNA. Clin Cancer Res. 2023;29(9):1689–97. https://doi.org/10.1158/1078-0432.CCR-22-2292

5 Tombolan L, Rossi E, Binatti A, Zin A, Manicone M, Facchinetti A, et al. Clinical significance of circulating tumor cells and cell-free DNA in pediatric rhabdomyosarcoma. Mol Oncol. 2022;16(10):2071–85. https://doi.org/10.1002/1878-0261.13197
6 Eisenhardt AE, Brugger Z, Lausch U, Kiefer J, Zeller J, Runkel A, et al. Genotyping of circulating free DNA enables monitoring of tumor dynamics in synovial sarcomas. Cancers. 2022;14(9):2078. https://doi.org/10.3390/cancers14092078
7 Krumbholz M, Eiblwieser J, Ranft A, Zierk J, Schmidkonz C, Stütz AM, et al. Quantification of translocation-specific ctDNA provides an integrating parameter for early assessment of treatment response and risk stratification in ewing sarcoma. Clin Cancer Res. 2021;27(21):5922–30. https://doi.org/10.1158/1078-0432.CCR-21-1324
8 Shulman DS, Klega K, Imamovic-Tuco A, Clapp A, Nag A, Thorner AR, 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):615–21. https://doi.org/10.1038/s41416-018-0212-9
9 Schmidkonz C, Krumbholz M, Atzinger A, Cordes M, Goetz TI, Prante O, et al. Assessment of treatment responses in children and adolescents with Ewing sarcoma with metabolic tumor parameters derived from 18F-FDG-PET/CT and circulating tumor DNA. Eur J Nucl Med Mol Imaging. 2020;47(6):1564–75. https://doi.org/10.1007/s00259-019-04649-1
10 Braig D, Becherer C, Bickert C, Braig M, Claus R, Eisenhardt AE, et al. Genotyping of circulating cell-free DNA enables noninvasive tumor detection in myxoid liposarcomas. Int J Cancer. 2019;145(4):1148–61. https://doi.org/10.1002/ijc.32216
11 Abbou S, Finstuen-Magro S, McDannell B, Feenstra M, Ward A, Shulman DS, et al. Rapid and highly sensitive approach for multiplexed somatic fusion detection. Mod Pathol. 2022;35(8):1022–33. https://doi.org/10.1038/s41379-022-01058-y
12 Seidel MG, Kashofer K, Moser T, Thueringer A, Liegl-Atzwanger B, Leithner A, et al. Clinical implementation of plasma cell-free circulating tumor DNA quantification by digital droplet PCR for the monitoring of Ewing sarcoma in children and adolescents. Front Pediatr. 2022;10:926405. https://doi.org/10.3389/fped.2022.926405
13 Stegmaier S, Sparber-Sauer M, Aakcha-Rudel E, Münch P, Reeh T, Feuchtgruber S, et al. Fusion transcripts as liquid biopsy markers in alveolar rhabdomyosarcoma and synovial sarcoma: a report of the Cooperative Weichteilsarkom Studiengruppe (CWS). Pediatr Blood Cancer. 2022;69(9):e29652. https://doi.org/10.1002/pbc.29652
14 Rostami A, Lambie M, Yu CW, Stambolic V, Waldron JN, Bratman SV. Senescence, necrosis, and apoptosis govern circulating cell-free DNA release kinetics. Cell Rep. 2020;31(13):107830. https://doi.org/10.1016/j.celrep.2020.107830
15 Bodlak A, Chang K, Channel J, Treece AL, Donaldson N, Cost CR, et al. Circulating plasma tumor DNA is superior to plasma tumor RNA detection in ewing sarcoma patients: ptDNA and ptRNA in ewing sarcoma. J Mol Diagn. 2021;23(7):872–81. https://doi.org/10.1016/j.jmoldx.2021.04.003
16 Abbou SD, Shulman DS, DuBois SG, Crompton BD. Assessment of circulating tumor DNA in pediatric solid tumors: the promise of liquid biopsies. Pediatr Blood Cancer. 2019;66(5):e27595. https://doi.org/10.1002/pbc.27595
17 Anderson CJ, Yang H, Parsons J, Ahrens WA, Jagosky MH, Hsu JH, et al. Can a liquid biopsy detect circulating tumor DNA with low-passage whole-genome sequencing in patients with a sarcoma? A pilot evaluation. Clin Orthop. 2024;483(1):39–48. https://doi.org/10.1097/CORR.0000000000003161
18 Christodoulou E, Yellapantula V, O'Halloran K, Xu L, Berry JL, Cotter JA, et al. Combined low-pass whole genome and targeted sequencing in liquid biopsies for pediatric solid tumors. NPJ Precis Oncol. 2023;7(1):21. https://doi.org/10.1038/s41698-023-00357-0
19 Calderillo-Ruíz G, Pérez-Yepez EA, García-Gámez MA, Millan-Catalan O, Díaz-Romero C, Ugalde-Silva P, et al. Genomic profiling in GIST: implications in clinical outcome and future challenges. Neoplasia. 2024;48:100959. https://doi.org/10.1016/j.neo.2023.100959

Supplementary Table 1. All possible translocation-associated tumor entities with chromosomal translocations for the study. Only six (6) of these entities were present at our clinic. All fusions and entities that occurred in our study are underlined.

Tumor	Translocation	Fusion product
Alveolar rhabdomyosarcoma	t(2;13)(q35;q14)	PAX3::FOXO1A
	t(1;13)(p36;q14)	PAX7::FOXO1A
Alveolar soft part sarcoma	t(X;17)(p11;q25)	TFE3::ASPL
Angiomatoid fibrous histiocytoma	t(12;16)(q13;p11)	FUS::ATF1
Clear cell sarcoma	t(12;22)(q13;q12)	EWSR1::ATF1
Congenital fibrocarsoma / mesoblastic nephroma	t(12;15)(p13;25)	ETV6::NTRK3
Dermatofibrosarcoma protuberans	t(17;22)(q22;q13)	COL1A1::PDGFB
Desmoplastic small-round cell tumor	t(11;22)(p13;q12)	EWS::WT1
Endometrial stromal sarcoma	t(7;17)(p15;q12)	JAZF1::JJAZ1
Ewing sarcoma / peripheral primitive neuroectodermal tumor	t(11;22)(q24;q12)	EWSR1::FLI1
	t(21;22)(q22;q12)	EWSR1::ERG
	t(7;22)(p22;q12)	EWSR1::ETV1
	t(17;22)(q12;q12)	EWSR1::E1AF
	t(2;22)(q33;q12)	EWSR1::FEV
Ewing-like sarcomas	t(4;19)(q53;q13), t(10;19)(q26;q13)	CIC::DUX4
	t(20;22) (q13.2;q12.2)	EWSR1::NFATC2
Extraskeletal myxoid chondrosarcoma	t(9;22)(q22;q12)	EWSR1::NR4A3

	t(9;17)(q22;q11)	RBP56::NR4A3
	t(9;15)(q22;q21)	TCF12::NR4A3
Fibromyxoid sarcoma, low grade	t(7;16)(q33;p11)	FUS::CREB3L2
	t(11;16)(p11;p11)	FUS::CREB3L1 (rare)
Inflammatory myofibroblastic tumor	t(1;2)(q22;p23)	TPM3::ALK
	t(2;19)(p23;p13)	TPM4::ALK
	t(2;17)(p23;q23)	CLTC::ALK
	t(2;2)(p23;q13)	RANBP2::ALK
Myxoid liposarcoma	t(12;16)(q13;p11)	FUS::DDIT3
	t(12;22)(q13;q12)	EWS::DDIT3
Synovial sarcoma	t(X;18)(p11;q11)	SS18::SSX1
		SS18::SSX2
		SS18::SSX4 (rare)

Supplementary Table 2: Number of liquid biopsy samples taken for ctDNA detection with the correlating event at time of sampling. During an event, 110 of the total 285 samples were taken, while the remaining 175 were taken during the routine follow-up. Abbreviations: adjuvant chemotherapy (adCT), neoadjuvant chemotherapy (nadCT), second-line chemotherapy (sLCT), third-line chemotherapy (tLCT), radiation (RTX), high-dose therapy (HDT), proton therapy (PT), targeted therapy (TT), high dose ifosfamide (HD-IFO).

Event at time of liquid biopsy	Number of LB samples for ctDNA detection taken in patients since initial diagnosis	Number of LB samples taken for ctDNA detection in patients since recurrence	Total:
adCT	19	0	19
nadCT	38	0	38
sLCT	5	2	7
tLCT	14	0	14
Surgery	6	0	6
RTX	1	0	1
Multilocular recurrence	9	5	14
Unilocular recurrence	2	1	3
HDT	1	1	2
PT	1	0	1
TT	0	3	3
HD-IFO	0	2	2
Total:	96	14	110