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Prognostic potential of fusion gene analysis using plasma cell-free RNA in malignant bone and soft tissue tumours

Naoki Furukawa[1,2], Nobuhiko Hasegawa[1*], Daisuke Kubota[1,2], Yasuhiro Nakamura[1], Hirokazu Tanaka[1,2], Shintaro Iwata[3], Akira Kawai[3], Tsuyoshi Saito[4], Tatsuya Takagi[1,2,5], Shinji Kohsaka[6] and Muneaki Ishijima[1,2,5]

Abstract

Background Liquid biopsy, which facilitates minimally invasive analysis of body fluid samples, has considerable potential as a diagnostic and prognostic tool in various cancers. Analysis of circulating tumour cells, circulating tumour DNA, and exosomes in liquid biopsies has advantages and disadvantages. However, their utility in rare cancers, such as malignant bone and soft tissue tumours, remains unknown. In this study, we examined the levels of circulating cell-free tumour RNA (cfRNA) in the blood of patients with malignant bone and soft tissue tumours harbouring specific fusion genes, to explore the relationship between fusion gene expression in the blood and therapeutic response and disease status, and to validate the clinical utility of liquid biopsy.

Methods The study involved 3 cases (7 samples) of Ewing's sarcoma, 6 cases (12 samples) of myxoid liposarcoma, and 1 case (2 samples) of synovial sarcoma with specific fusion genes. Fusion gene analysis was performed using tumour tissue samples to identify breakpoints. Primers for liquid biopsy were designed based on the fusion genes identified. cfRNA was extracted from each patient's plasma and used for reverse transcription polymerase chain reaction (RT-PCR) with the designed primers. The RT-PCR product was subjected to Sanger sequencing.

Results Fusion gene breakpoints were identified in 10 samples from 6 cases. The fusion gene detection rate in the blood was 100% at both naïve status and symptom exacerbation in patients with Stage IV disease. In patients with Stage III disease progressing to Stage IV, the fusion gene was detected in the blood prior to imaging tests.

Conclusions The detection of specific fusion genes from cfRNAs shows potential for monitoring the progression of fusion-related sarcomas in the context of chemotherapy.

Keywords Cell-free RNA, Fusion gene, Liquid biopsy, Malignant bone and soft tissue tumour

*Correspondence:
Nobuhiko Hasegawa
n-hasegawa@juntendo.ac.jp
[1] Department of Orthopaedics, Faculty of Medicine, Juntendo University, Tokyo 113-8421, Japan
[2] Department of Medicine for Orthopaedics and Motor Organ, Juntendo University Graduate School of Medicine, Tokyo, Japan
[3] Department of Musculoskeletal Oncology and Rehabilitation Medicine, National Cancer Center Hospital, Tokyo, Japan
[4] Department of Human Pathology, Juntendo University School of Medicine, Tokyo, Japan
[5] Department of Community Medicine and Research in Bone and Joint Diseases, Juntendo University Graduate School of Medicine, Tokyo, Japan
[6] Division of Cellular Signaling, National Cancer Center Research Institute, Tokyo, Japan

Background

Bone and soft tissue tumours arise in bone and mesenchymal tissues, such as fat, nerves, and blood vessels. Clinically, they are classified as primary or metastatic. Primary malignant bone and soft tissue tumours account for approximately 1% of all cancers. These are primarily sarcomas with over 70 subtypes. Given the rarity, histological diversity, and refractoriness of the disease, many patients have a poor prognosis upon diagnosis. The overall 5-year relative survival rate is approximately 50%, with 16% of patients already presenting metastases at diagnosis, in which case, the 3-year survival rate is just 20%–25% [1, 2].

The pathological diagnosis of malignant bone and soft tissue tumours is often complex and challenging. Currently, a definitive diagnosis of malignant bone and soft tissue tumours relies on tissue biopsy and imaging evaluation. Particularly, tissue biopsy has emerged as the gold standard for diagnosis. However, tissue biopsy is invasive and difficult to perform depending on disease location [3]. Furthermore, it requires a considerable amount of time to accurately diagnose the condition. In a previous study, genetic assessment resulted in a change in the pathologic diagnosis of 14% of cases [4].

No guidelines regarding the method and duration for determining malignant bone and soft tissue tumour treatment efficacy have been established, with no clear standards for postoperative chemotherapy administration [5, 6]. Therefore, it is imperative to develop markers that can serve as diagnostic tools and biomarkers to determine treatment efficacy and elucidate the underlying pathophysiology, in order to advance disease management.

Recently, liquid biopsy has garnered interest as a non-invasive diagnostic approach that can be performed to analyse circulating tumour cells (CTCs) and nucleic acids released from the tumour (circulating tumour DNA [ctDNA], circulating tumour RNA, and extracellular microRNA) present in body fluids, including blood and urine. Liquid biopsy facilitates minimally invasive and time-course monitoring using body fluid samples [7].

Previous liquid biopsy studies using blood specimens and CTCs have shown that CTC detection almost 5 years after the initial diagnosis predicts late recurrence in patients with operable hormone receptor-negative breast cancer [8]. In non-metastatic colorectal cancer, the detection of CTCs 2–3 years after surgery predicted a poor prognosis [9]. However, the usefulness of liquid biopsy in malignant bone and soft tissue tumours has not yet been established [3, 10].

CTCs are tumour cells released from the primary tumour site and circulating through the bloodstream, potentially reflecting metastasis. As these cells are not apoptotic, CTCs reflect various characteristics of tumour cells. However, CTCs are present at extremely low levels in the peripheral blood of patients with cancer (1 cell per 1 mL of blood). Although various detection systems have been developed, the results are not robust [11, 12]. cfDNA is particularly useful in mutation analysis, with a match rate of more than 70% with tissue DNA in non-small cell lung cancer (NSCLC) [13]. However, the fusion gene detection rate based on cfDNA is reportedly lower than that achieved based on RNA [14, 15]. One reason is that gene fusions usually occur in introns, which often contain repetitive sequences, making it difficult to detect intron fusion points with high sensitivity using DNA capture probes [16, 17].

We previously compared cfDNA and cfRNA obtained from the blood samples of patients with lung cancer and found that cfRNA (78%) was more effective for fusion gene analysis than cfDNA (33%) [18].

Chromosomal translocations have been found in approximately 30% of sarcomas, and several fusion genes have been identified as possible causative genes [19]. If these fusion genes can be detected as tumour-specific factors, through both histological diagnosis and liquid biopsy, they have the potential to serve as minimally invasive tools for the diagnosis and monitoring of disease. Therefore, the analysis of cfRNA through liquid biopsy may be appropriate.

In this study, we investigated whether fusion genes detected in malignant bone and soft tissue tumour specimens could be detected in blood-derived cfRNA.

Methods

Case Selection

We collected 21 specimens from 10 patients diagnosed with Ewing's sarcoma, myxoid liposarcoma, or synovial sarcoma at Juntendo University Hospital. Patients undergoing biopsy or surgery from whom a specimen could be obtained and all tumours that tested positive for *EWSR1::FLI1*, *FUS::DDIT3*, or *SS18::SSX1* through RT-PCR were considered potentially eligible. Those lost to follow-up or whose diagnosis was changed were excluded. Ewing's sarcoma, myxoid liposarcoma, and synovial sarcoma were diagnosed based on the histological and immunohistochemical characteristics of samples collected via tissue biopsy or surgery. Using tissue RNA, all tumour samples were confirmed to contain tissue-specific fusion genes. Blood samples were collected before surgery or chemotherapy and again after chemotherapy. Blood samples (10–15 mL) were collected in blood collection tubes (EDTA-2 K) and centrifuged within 1 h after collection. Only plasma samples were used as specimens; they were centrifuged and stored at − 80 °C.

All patients underwent tumour imaging and subsequent assessment every 3–6 months until disease

progression. The tumour response to treatment was also evaluated. Ewing's sarcoma was treated using VDC-IE therapy (comprising vincristine, doxorubicin, cyclophosphamide, ifosfamide, and etoposide) or doxorubicin (DOX) monotherapy [20]. Myxoid liposarcoma was treated with either AI therapy (which consists of doxorubicin and ifosfamide) or DOX monotherapy [21]. Synovial sarcoma was treated exclusively with AI therapy [22]. The study protocol was approved by the Ethics Committee of Juntendo University Hospital (approval no. E22-0181-H01). Written informed consent was obtained from all patients. All procedures involving human participants were performed in accordance with the ethical standards of the Institutional Research Committee, the 1964 Declaration of Helsinki, and subsequent revisions or equivalent ethical standards.

RNA Extraction from Tumour Tissue and Fusion Gene Diagnosis

RNA was extracted from 2 mm^3 frozen or formalin-fixed paraffin-embedded (FFPE) surgical specimens using an RNeasy Plus Mini Kit or RNeasy FFPE Kit (QIAGEN, Germany).

To identify the breakpoint of fusion genes in each surgical specimen, RNA quality was first determined using NanoDrop Lite (Thermo Fisher Scientific, Waltham, MA, USA), and total RNA was reverse-transcribed using SuperScript IV VILO Master Mix (Thermo Fisher Scientific). Reverse transcription polymerase chain reaction (RT-PCR) was performed using Go Tag G2 Green Master Mix (Promega Corp., Madison, WI, USA) at an annealing temperature of 50 °C and 30 cycles using primer pairs for *EWSR1::FLI1*, *FUS::DDIT3*, and *SS18::SSX1* (primer sequences are outlined in Additional file 1).

The samples generated via RT-PCR were subjected to Sanger sequencing to identify fusion gene breakpoints.

Extraction and RT-PCR of cfRNA

cfRNA was extracted from approximately 4–5 mL of whole plasma using the Quiq-cfRNA Serum and Plasma Kit (Zymo Research, Irvine, CA, USA). For RNA quality evaluation, RIN value and DV200 of the cfRNA were measured using a 4150 TapeStation (Agilent Technologies, Santa Clara, CA, USA). Two microliters of extracted RNA was reverse-transcribed to cDNA using the GenNext RamDA-seq Single Cell Kit (TOYOBO, Japan) and NSR Primer Set for humans (TOYOBO). The resulting 9 µL of template cDNA was PCR-amplified using Go Tag G2 Green Master Mix (Promega Corp.), and the same primer pairs were used for fusion gene detection in tissue samples. The samples generated via RT-PCR were subjected to Sanger sequencing to identify fusion gene breakpoints.

A breakpoint was defined when at least 10 bases were identified between the breakpoints.

Statistical analysis

Graphs were generated and analysed using Prism Ten software (GraphPad). Statistical significance was determined using a two-tailed unpaired t-test, with p values less than 0.05 being considered significant.

Results

Protocol for Identifying Fusion Genes in Malignant Bone and Soft Tissue Tumours

The sensitivity of fusion gene analysis using cfRNA depends on cDNA synthesis efficiency. Therefore, the process from cDNA synthesis to RT-PCR was optimised. An amplicon-based method was used to enrich the target fusion points via PCR amplification with specific primers, which required less input cDNA. Figure 1 shows an overview of the proposed cfRNA-based fusion gene detection assay. The GenNext RamDA-seq Single Cell Kit (TOYOBO) was used for cDNA synthesis.

Clinical Information and Amount of RNA Extracted for Each Patient

The characteristics of the 10 patients included in the study are listed in Table 1. Clinical information for each patient, the presence or absence of a fusion gene, the amount of cfRNA extracted from plasma, and its RIN and DV200 values are shown in Additional file 2. The mean ± SD of RIN and DV200 of the cfRNA were 4.56 ± 0.84 and 60.78 ± 7.82, respectively. These data are presented separately for Ewing's sarcoma, myxoid liposarcoma, and synovial sarcoma samples in Additional file 3. The relationship between patient status (naïve/progressive disease [PD] vs. no evidence of disease [NED]/stable disease [SD]/partial response [PR]) and cfRNA collection was examined; however, no significant difference in cfRNA yield according to patient status was observed (Additional file 4a).

Detection of Fusion Genes in Plasma cfRNA

Of the 21 cfRNA samples validated, 47.6% (10/21 samples) contained fusion genes (Table 2). Fusion genes were detected in 57.1% (4/7 samples) of Ewing's sarcoma, 50% (6/12 samples) of myxoid liposarcoma, and 0% (0/2 samples) of synovial sarcoma samples.

Fusion genes were detected in 0% (0/2 samples) of Stage III and 80% (4/5 samples) of Stage IV Ewing's sarcoma samples; 60% (3/5 samples) of Stage II, 25% (1/4 samples) of Stage III, and 67% (2/3 samples) of Stage IV myxoid liposarcoma samples. Both synovial sarcoma samples were from Stage II patients, with neither revealing breakpoints. Fusion genes were found in 100% (5/5

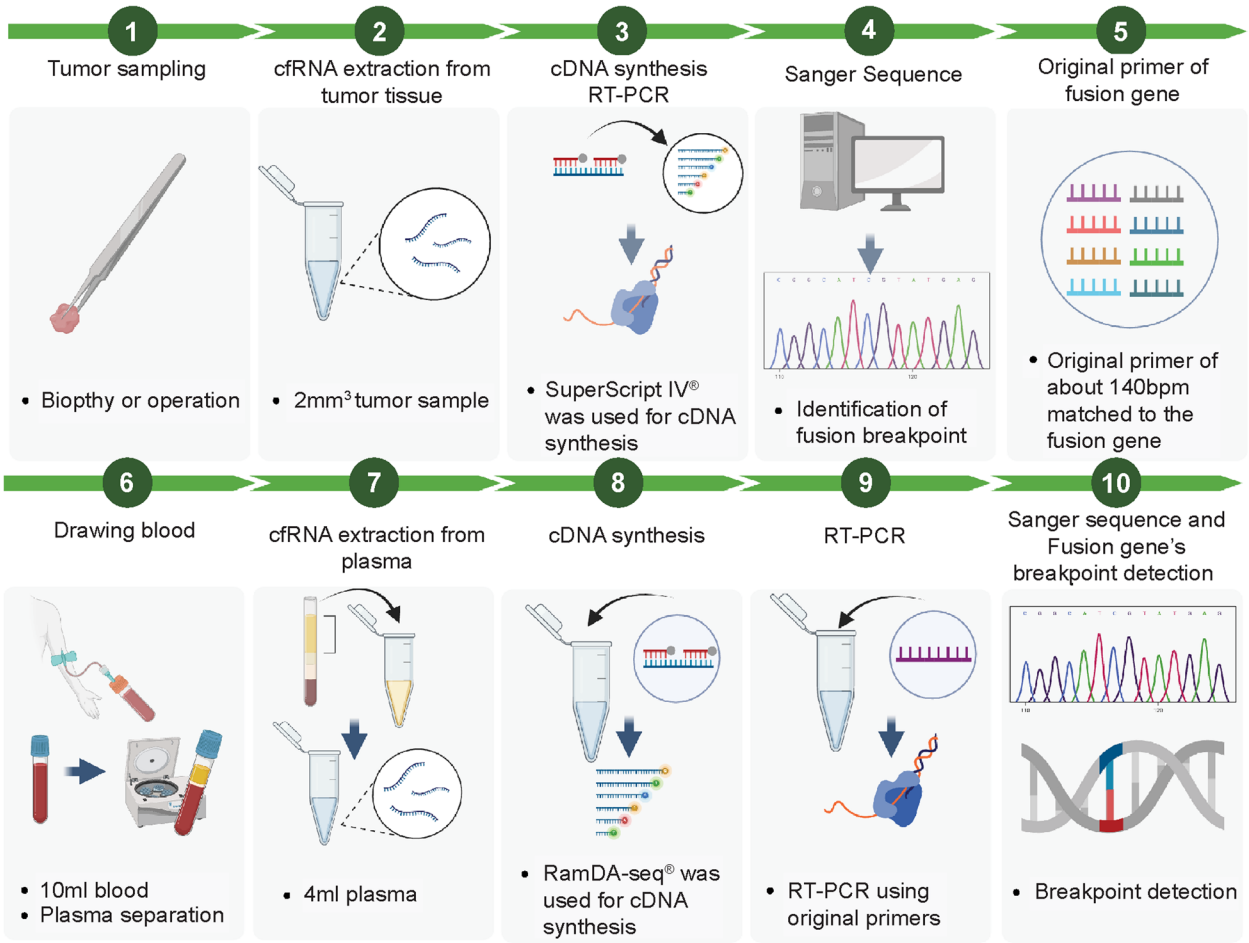

Fig. 1 Procedure for detecting fusion genes in the blood. Extract circulating tumour RNA (cfRNA) from 2-mm^3 tumour samples, perform reverse transcription polymerase chain reaction (RT-PCR), and confirm breakpoint via Sanger sequencing analysis. Design original primers that match the breakpoint found in the fusion genes of the tumour. Centrifuge 10 mL of the patient blood and extract 4 mL of plasma. Extract cfRNA from the plasma, then perform RT-PCR and Sanger sequencing using the original primers. Confirm that the same fusion gene is detected from the cfRNA as found in the tumour. Illustrations were created using BioRender, and permission was obtained

samples) of Ewing's sarcoma and myxoid liposarcoma Stage IV cases that were at naïve or progressive disease status (Fig. 2).

Fusion Gene Detection by Tumour Diameter

Of the untreated tumours, 40% (2/5 samples) were between 50 and 100 mm, 50% (1/2 samples) were between 100 and 200 mm, and 33.3% (1/3 samples) were > 200 mm in diameter. No significant association of tumour size with fusion gene detection rate was found (Additional file 4b).

Evaluation of Quality by Specimen Type

We analysed four cases of myxoid liposarcoma using available residual samples. Three cases (#4, #5, and #8) were examined using frozen tissue, while an FFPE sample was used in one case (#7). The cfRNA comparison samples used consisted of the four samples in which the fusion gene could be identified. No significant difference was detected between cfRNA and frozen samples; however, a difference was observed between cfRNA and FFPE (Additional files 5 and 6).

Case-Specific Findings

In this study, we show the relationship between cfRNA fusion gene expression and clinical status in three cases of Ewing's sarcoma, six cases of myxoid liposarcoma, and one case of synovial sarcoma.

Case 2 was of a patient with Ewing's sarcoma (Stage IV), who had spinal and brain metastases before treatment. The primary tumour was in the right iliac bone. Fusion genes were detected in the blood before treatment and up to the end of two courses of chemotherapy (one course doxorubicin alone and one course

Table 1 Demographic and clinicopathologic characteristics of the 10 patients enrolled in the study

Feature	No. of patients ($N=10$)
Median age, years (range)	38.2 (20–65)
Sex, N (%)	
Male	7 (70)
Female	3 (30)
T-stage, N (%)	
1	0 (0)
2	10 (100)
N-stage, N (%)	
X	0 (0)
0	10 (100)
1	0 (0)
M-stage, N (%)	
0	7 (70)
1	3 (30)
Clinical or pathologic stage, N (%)	
I	0 (0)
II	4 (40)
III	3 (30)
IV	3 (30)
Fusion genes in tissue, N (%)	
EWSR1::FLI1	3 (30)
FUS::DDIT3	6 (60)
SS18::SSX1	1 (10)
Initial treatment, N (%)	
Operation	5 (50)
Chemotherapy	5 (50)
Radiation	0 (0)

vincristine–ifosfamide). After three courses of vincristine–ifosfamide, the primary tumour shrank, and the fusion gene was temporarily undetectable. After another course of vincristine–ifosfamide, the disease worsened rapidly, and the fusion gene was detected again. The identified fusion was of *EWSR1* exon 7 and *FLI1* exon 6 (Fig. 3a and b).

Three myxoid liposarcoma cases (4 and 8) were of Stage II disease, and case 5 was of Stage III disease. Case 4 involved a soft tissue tumour of the left knee, diagnosed as a myxoid liposarcoma through needle biopsy, for which extensive tumour excision was planned. Fusion genes were detected in blood collected during surgery. The gene fusion was of *FUS* exon 7 and *DDIT3* exon 2 (Fig. 3c). The fusion genes were re-identified after four chemotherapy courses (Fig. 3d). Currently, the disease course is being followed. Case 5 involved a right femoral soft tissue tumour, diagnosed as myxoid liposarcoma through needle biopsy. No fusion gene was detected in the blood sample collected during surgery. However, a fusion of *FUS* exon 5 and *DDIT3* exon 2 was detected in the blood before postoperative chemotherapy (Fig. 3e). One course of doxorubicin–ifosfamide was administered, after which liver metastases developed. The fusion gene was still detected after three courses of doxorubicin–ifosfamide; however, it was no longer detectable after radiofrequency ablation of liver metastases (Fig. 3f). Case 8 involved a soft tissue tumour in the left thigh, diagnosed as myxoid liposarcoma through needle biopsy. Wide resection of the tumour was planned, and although no fusion gene was detected in the preoperative blood sample, it was detected in the blood after one course of postoperative doxorubicin–ifosfamide. The fusion genes identified were *FUS* exon5 and *DDIT3* exon 2 (Additional file 7a). Two additional courses of doxorubicin–ifosfamide were administered, with no evidence of recurrence to date (Additional file 7b). Case 7 involved Stage IV myxoid liposarcoma found in the left thigh and diagnosed through needle biopsy. Metastases to the chest wall and liver were present. Fusion genes were detected before chemotherapy (the treatment of choice, given the Stage IV status). The fusion genes identified were *FUS* exon 5 and *DDIT3* exon 2 (Additional file 7c and 7d).

Based on needle biopsy, a soft tissue tumour arising from the back of the hand in Case 10 was diagnosed as synovial sarcoma. To preserve hand function to the greatest extent possible, two courses of doxorubicin–ifosfamide were administered as preoperative chemotherapy, followed by wide resection of the tumour. Blood samples were collected during surgery, which revealed no fusion genes. Three courses of doxorubicin–ifosfamide were administered postoperatively, with no fusion genes detected in postoperative blood samples.

Discussion

Guardant Reveal™ has been used clinically for early cancer detection and post-treatment recurrence risk assessment and recurrence monitoring. However, its indications are limited to lung, breast, and colorectal cancers, with no clinical application in sarcoma. We sought to develop an assay that could determine the histological type of sarcoma and evaluate disease status by identifying fusion genes through liquid biopsy. This approach could serve as an adjunct diagnostic tool for the detection of micrometastases that cannot be detected using diagnostic imaging after surgery. Fusion genes were identified in 10 samples from 6 cases. The fusion gene detection rate in the blood was 100% at naïve or progressive disease status in patients with Stage IV disease.

The amount of extracted cfRNA and efficiency of cDNA synthesis are directly related to fusion gene detection in specimens. Reverse transcription with random displacement amplification (RT-RamDA), which enables

Table 2 Summary of 10 cases analysed for fusion gene detection using the cfRNA-based assay

Case #	Sex	Age	Diagnosis	Stage	Tissue analysis				Treatment			Treatment at sample collection	Liquid fusion detection	
					Tumour size (mm)	Fusion	Method	Metastasis	1st	2nd	3rd		Status at sample collection	cfRNA
# 1	M	21	EwS	IV	83.6	*ESWR1::FLI1*	RT-PCR	Lung	CTx(VDC-IE)	-	-	Pre treatment	Naïve	Detected
# 2	M	20	EwS	IV	134.2	*ESWR1::FLI1*	RT-PCR	SpinalBrain	CTx(DOX)	RTx	CTx(VDC-IE)	Pre treatment	Naïve	Detected
												CTx 2 course	SD	Detected
												CTx 4 course	PR	ND
												CTx 5 course	PD	Detected
# 3	F	27	EwS	III	80.6	*ESWR1::FLI1*	RT-PCR	Non	CTx(VDC-IE)	-	-	CTx 2 course	PR	ND
												CTx 6 course	SD	ND
# 4	F	37	MLS	II	52.7	*FUS::DDIT3*	RT-PCR	Non	Op	CTx(AI)	-	Pre treatment	Naïve	Detected
												CTx 4 course	NED	Detected
# 5	M	57	MLS	III	246.2	*FUS::DDIT3*	RT-PCR	Non	Op	RTx	CTx(AI)	Pre treatment	Naïve	ND
												Pre CTx	NED	Detected
				IV				Liver				CTx 3 course	PD	Detected
												Post RFA	SD	ND
# 6	F	41	MLS	II	177.8	*FUS::DDIT3*	RT-PCR	Non	Op	-	-	Pre treatment	Naïve	ND
# 7	M	65	MLS	IV	207.9	*FUS::DDIT3*	RT-PCR	Chest wallLiver	CTx(DOX)	-	-	Pre treatment	Naïve	Detected
# 8	M	31	MLS	II	60.8	*FUS::DDIT3*	RT-PCR	Non	Op	CTx(AI)	-	Pre treatment	Naïve	ND
												Pre CTx	NED	Detected
# 9	M	50	MLS	III	205.8	*FUS::DDIT3*	RT-PCR	Non	CTx(AI)	Op	-	Pre treatment	Naïve	ND
												CTx 2 course	PD	ND
# 10	M	33	SS	II	57.9	*SS18::SSX1*	RT-PCR	Non	CTx(AI)	Op	-	Pre treatment	Naïve	ND
												CTx 5 course	NED	ND

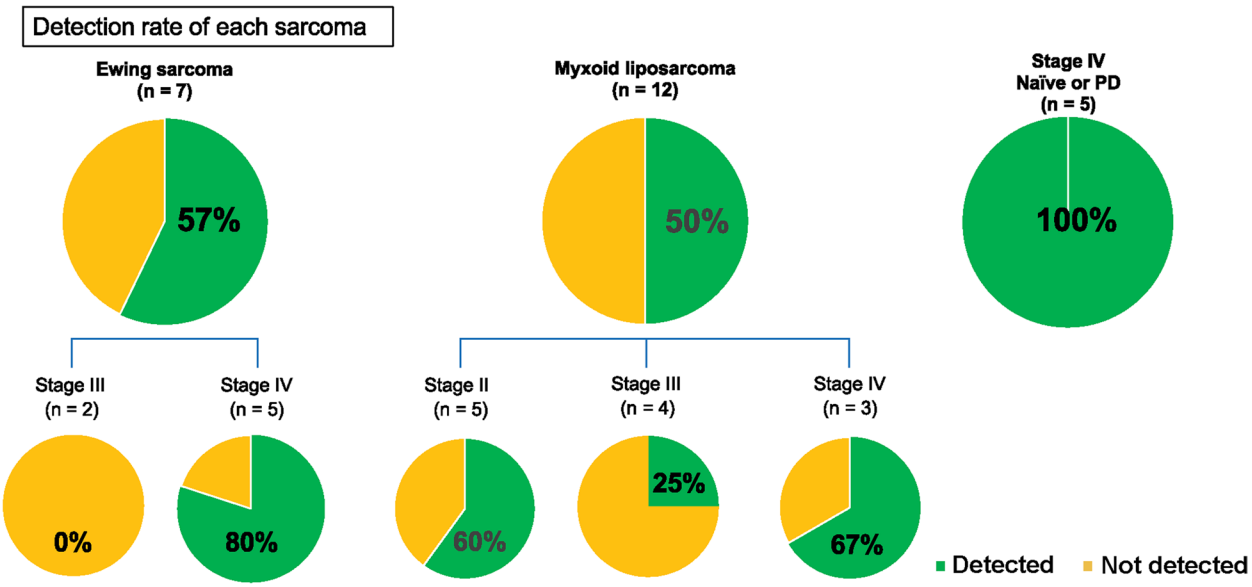

Fig. 2 Sensitivity of fusion gene detection for each tumour and cancer stage. In Ewing's sarcoma, the fusion transcript was detected only in Stage IV specimens. In myxoid liposarcoma, the fusion transcript was detected in Stage II, III, and IV specimens. The detection rate was 100% in Stage IV specimens at the time of naïve and progression disease status

cDNA synthesis from trace amounts of RNA according to the protocol for fusion gene detection assays established by Hasegawa et al. [18], was used in the present study. The GenNext RamDA-seq Single Cell Kit (TOYOBO) uses reverse transcription primers without common sequences, enabling cDNA synthesis without bias and analysis of even difficult-to-detect genes (rare genes and highly degraded genes), thereby suppressing cDNA synthesis from rRNA, enabling a higher amplification of target cDNA and the detection of low amounts [23].

When designing the original primers for breakpoint detection, we set a relatively short amplicon size of approximately 140 bp, which may be less affected by fragmented samples.

Sanger sequencing was performed as opposed to next-generation sequencing to confirm the breakpoints. Sanger sequencing is highly reliable for DNA read accuracy and facilitates accurate determination of the breakpoint and target gene. It has the potential to provide immediate confirmation of results in clinical practice. Additionally, DNA-based analysis requires the creation of primers suitable for long-distance introns, which is a complicated process. With the RNA-based method, fusion genes can be easily identified by focusing only on the exons. If this technique is effective in producing panels of multiple sarcoma fusion gene primers, it could potentially become a valuable diagnostic tool in the future.

The breakpoint of the fusion gene was found in four samples from two cases of Ewing's sarcoma and in six samples from four cases of myxoid liposarcoma. No fusion genes were detected in synovial sarcomas. In past reports, the fusion gene thought to be derived from cfRNA has been detected in the blood of patients with Ewing's sarcoma [24]. The method involved RT-qPCR for the identification of fusion genes. We identified the exact sequence of fusion genes via Sanger sequencing. In one case of Ewing's sarcoma where a fusion gene was not detected, the localised sarcoma progressed without metastasis. In the two cases in which fusion genes were detected, these were presumably detected in blood cfRNA with the occurrence of metastasis. The subsequent detection of the fusion gene upon worsening of the patient's clinical condition further supports this possibility. For Ewing's sarcoma, the ability to assess cfRNA in the blood may aid in the evaluation of treatment for metastasis.

To our knowledge, no previous report has described the cfRNA-based detection of fusion genes in patients with myxoid liposarcoma. In one case where the fusion gene was not detected preoperatively, it was detected in the blood cfRNA 7 months after surgery and postoperative radiation therapy. During this period, no metastases were detected on imaging; however, liver metastases were detected during subsequent follow-up. This finding suggests that blood cfRNA may be more sensitive for detecting metastases than imaging evaluation.

No fusion gene was detected in blood cfRNA of the patient with synovial sarcoma before treatment or after

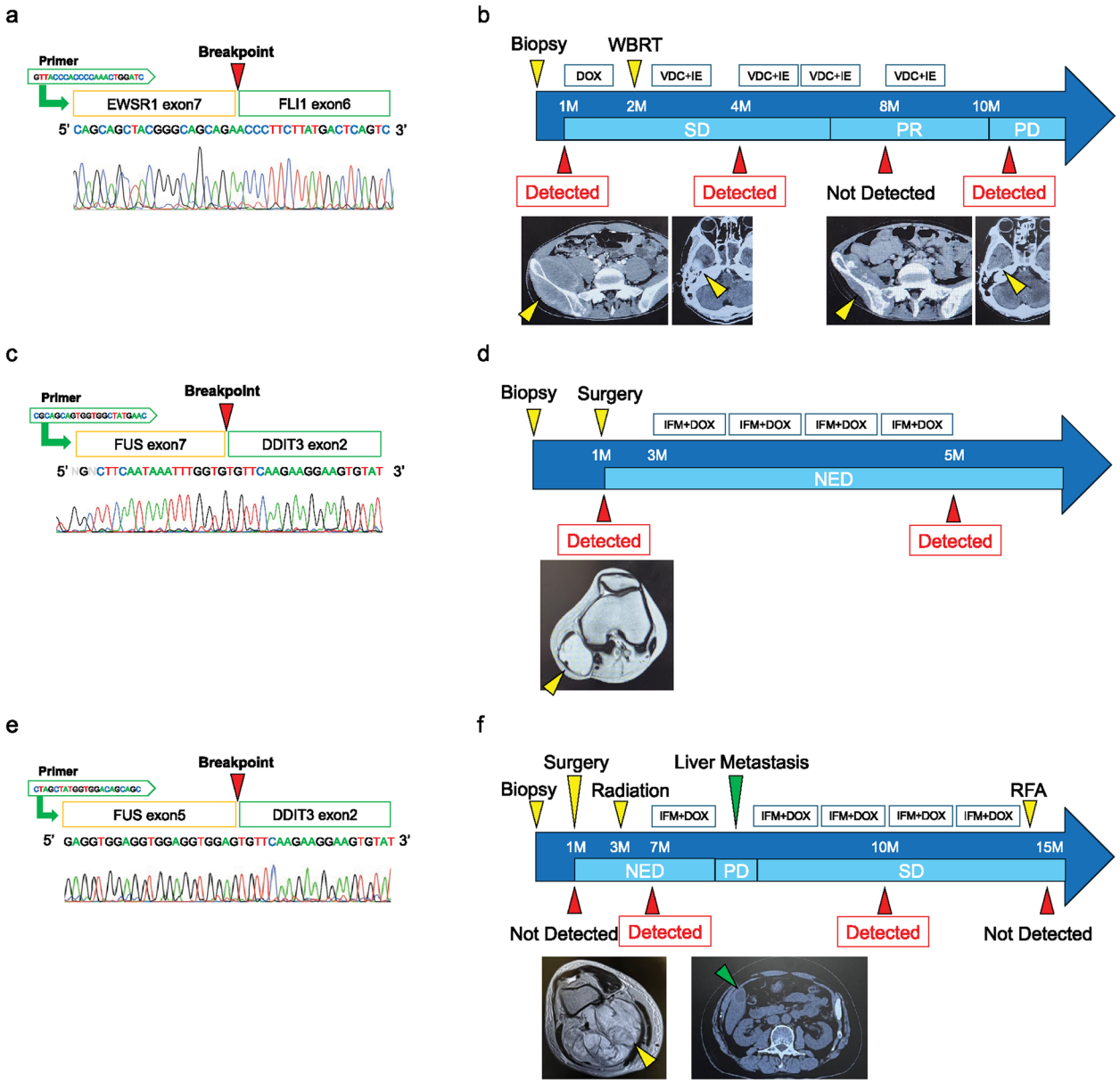

Fig. 3 Clinical cases. **a** EWSR1 (exon7)::FLI1 (exon6) fusion gene identified from blood samples using Sanger sequencing. **b** Case 2: A 20-year-old man with Ewing's sarcoma of the pelvis presented with metastases in the thoracic spine and brain. The fusion gene was detected in blood at the beginning of treatment and disappeared in response to treatment. The fusion gene was detected again as the disease worsened. **c** FUS (exon 7)::DDIT3 (exon 2) fusion gene identified from blood samples using Sanger sequencing. **d** Case 4: A 37-year-old woman with myxoid liposarcoma of the left knee. Prior to operation, the tumour was of Stage II. Extensive resection was performed. The fusion gene was detected in a preoperative blood sample and in a blood sample after four courses of chemotherapy (AI), but no evidence of recurrence was found. **e** FUS (exon5)::DDIT3 (exon2) fusion gene identified from blood samples using Sanger sequencing. **f** Case 5: A 57-year-old man with myxoid liposarcoma of the right thigh. Prior to operation, the patient had a Stage III disease, and no fusion genes were detected in the preoperative blood sample. Extensive resection was performed, and the fusion gene was detected in the blood just before starting postoperative chemotherapy, with liver metastasis detected in a subsequent examination. After radiofrequency ablation for liver metastasis, the fusion transcript was not detected in the blood

extensive tumour resection and chemotherapy. It has been reported that 13% of patients with synovial sarcoma have distant metastases at diagnosis. In this case, as metastases were not detected before treatment, it is possible that the fusion transcript had not yet disseminated into the blood [22]. The detection rate of *SS18::SSX1/2* in the blood circulation is reportedly low and thus markers other than tumour type-specific fusion

transcripts should be considered [25]. Future validation is expected to also reveal sarcomas that are more suitable for fusion gene detection in the blood.

The potential clinical utility of CTCs and ctDNA in sarcoma has been reported. In a study of sarcoma using ctDNA, a correlation was found between the allele fraction of ctDNA and responses to combined immunotherapy and local cryotherapy for unresectable or metastatic soft-tissue tumours [26]. Other studies have reported that HK2, as a metabolic function-related marker, is an indicator for CTC detection in sarcomas, showing high consistency between prediction and clinical results in post-treatment evaluation and disease-free survival [27]. Blood monitoring holds considerable potential in sarcoma. However, CTC-based detection of *EWS::FLI1* fusion transcripts in Ewing's sarcoma and *SS18::SSX1* in synovial sarcoma has shown lower sensitivity [25, 28]. Plasma cfDNA sequencing in patients with advanced NSCLC showed relatively high sensitivity for detecting gene mutations but low sensitivity for gene fusions [13]. Utilising cfRNA, the technique used in this study is simple and has potential to reveal disease status. The benefit of postoperative chemotherapy for sarcomas such as myxoid liposarcomas has not been clearly defined, and the first choice of treatment is extensive resection. Currently, chemotherapy is administered prophylactically at the physician's discretion, with consideration of age, social background, pathology, and other factors. According to a recent report, systemic inflammatory indices play a remarkable role in patients with sarcoma. This study suggests that these indices may serve as prognostic markers for STS and highlights the importance of the lymphocyte-to-monocyte ratio (LMR) as a potential predictor of trabectedin treatment efficacy. Future validation of multiple biomarkers, including cfRNA, will be crucial to elucidate their clinical relevance and potential use in sarcoma management [29]. The identification of sarcoma-related factors in the blood can be an indicator for earlier postoperative chemotherapy than the detection of recurrent metastases by imaging diagnostics, enabling treatment initiation at the appropriate time.

This study had some limitations. Blood samples were collected according to treatment timing. However, owing to the limited amount of blood collected at one time, increasing the amount of extracted RNA by increasing the blood collection volume was challenging. This was a single-centre study, and involved issues such as the small sample size and the variability in sample collection timing. This was a pilot study, and further verification at other facilities is needed to validate the efficacy of the protocol.

In some cases of Naive and PD, fusion genes have not been identified from cfRNA. Although the verification of RIN was only conducted in a limited number of cases, there was no significant difference between frozen tissue and cfRNA, whereas there was a difference in quality when compared with FFPE. Even with poor RNA quality, such as in the case of FFPE, fusion genes can still be detected. One possible issue that may arise from the inability to identify cfRNA is the PCR amplification bias of non-tumour RNA present in the plasma. In the future, it will be necessary to develop methods for cDNA synthesis and PCR that can effectively amplify small quantities of tumour-derived RNA. Additionally, further validation of our findings is imperative because malignant bone and soft tissue tumours are rare cancers, resulting in a small cohort.

Conclusions

In conclusion, we showed that cfRNA can be used to detect sarcoma-specific fusion genes from liquid biopsies. Although some studies have used CTCs and ctDNA as biomarkers for monitoring disease status in patients with sarcoma, this approach is particularly effective in detecting fusion genes with high sensitivity, and may further assist in determining the most appropriate treatment strategy.

Abbreviations

cfRNA	Cell-free tumour RNA
CTC	Circulating tumour cell
ctDNA	Circulating tumour DNA
CTx	Chemotherapy
EwS	Ewing's sarcoma
FFPE	Formalin-fixed paraffin-embedded
MLS	Myxoid liposarcoma
NA	Not analysed
NED	No evidence of disease
NSCLC	Non-small cell lung cancer
Op	Surgery
PD	Progressive disease
PR	Partial response
RT-PCR	Reverse transcription polymerase chain reaction
RT-RamDA	Reverse transcription with random displacement amplification
RTx	Radiation
SD	Stable disease
SS	Synovial sarcoma

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12885-025-13950-2.

Additional file 1: Supplementary Table S1. PCR Primers for fusion gene detection.

Additional file 2: Supplementary Table S2. RIN value, DV200, and cfRNA concentration in individual cases.

Additional file 3: Supplementary Table S3. RIN value, DV200, and cfRNA concentration in each sarcoma type.

Additional file 4: Supplementary Figure S1. (a) Relationship between the amount of circulating tumour RNA (cfRNA) extracted and the patient's clinical condition. A comparison of 11 naïve and progressive disease (PD) samples and 9 no evidence of disease (NED), stable disease (SD), and partial response (PR) samples revealed no significant difference in the

extracted cfRNA amount, particularly in relation to the patients' clinical condition. The statistical significance of the data was determined using a two-tailed unpaired t-test, with p values less than 0.05 considered as significant. n.s., not significant. (b) Relationship between tumour size and fusion gene detection. No significant difference between tumour size and fusion gene detection was found.

Additional file 5: Supplementary Figure S2. (a) Comparison of cfRNA and frozen tissue RIN values for each sample. Statistical significance was determined using a two-tailed unpaired t-test, with p values less than 0.05 considered significant. n.s., not significant. (b) Comparison of cfRNA and FFPE RIN values for #7. (c) Comparison of the average RIN values of three samples each of cfRNA and frozen tissue sample.

Additional file 6: Supplementary Table S4. RIN and DV200 values in frozen tissue, FFPE, and cfRNA in MLS.

Additional file 7: Supplementary Figure S3. (a) FUS (exon 5)/DDIT3 (exon 2) fusion gene identified by Sanger sequencing from blood samples. (b) Case 8: A 31-year-old male with left femoral myxoid liposarcoma. No fusion genes were detected in the preoperative blood sample of the patient with Stage II disease. Extensive resection was performed. A fusion gene was detected in the blood after one course of postoperative chemotherapy. The tumour did not metastasise to any specific region. (c) FUS (exon 5)/DDIT3 (exon 2) fusion gene identified by Sanger sequencing from blood samples. (d) Case 7: A 65-year-old male with a myxoid liposarcoma of the left thigh. Stage IV cancer, with chest wall and liver metastasis, were observed at the time of diagnosis. A fusion gene was detected in his blood before the start of chemotherapy.

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Authors' contributions
F.N. contributed to the design and execution of experiments, data analysis, and figures construction, and wrote the paper. D.K. and N.H. analyzed and interpreted the data. D.K, Y.N, H.T, S.I, A.K, T.S, T.T, S.K, and M.I contributed to the sample collection and reviewed the manuscript. N.H. contributed to the conceptualization, supervision, and design of experiments, and wrote and edited the paper.

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Data availability
The datasets generated and/or analysed during the current study are available in the NBDC repository (hum0499).

Declarations

Ethics approval and consent to participate
The study protocol was approved by the Ethics Committee of Juntendo University Hospital (approval no. E22-0181-H01). All procedures involving human participants were performed in accordance with the ethical standards of the Institutional Research Committee, the 1964 Declaration of Helsinki, and subsequent revisions or equivalent ethical standards. Written informed consent was obtained from all patients.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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