Genomic EWSR1 Fusion Sequence as Highly Sensitive and Dynamic Plasma Tumor Marker in Ewing Sarcoma

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Abstract

Purpose: The application of the tumor-specific genomic fusion sequence as noninvasive biomarker for therapy monitoring in Ewing sarcoma (EwS) has been evaluated.

Experimental Design: EwS xenograft mouse models were used to explore detectability in small plasma volumes and correlation of genomic EWSR1-FLI1 copy numbers with tumor burden. Furthermore, 234 blood samples from 20 EwS patients were analyzed before and during multimodal treatment. EWSR1 fusion sequence levels in patients’ plasma were quantified using droplet digital PCR and compared with tumor volumes calculated from MRI or CT imaging studies.

Results: Kinetics of EWSR1 fusion sequence copy numbers in the plasma are correlated with changes of the tumor volume in patients with localized and metastatic disease. The majority of patients showed a fast reduction of cell-free tumor DNA (ctDNA) during initial chemotherapy. Recurrence of increasing ctDNA levels signaled relapse development.

Conclusions: Genomic fusion sequences represent promising noninvasive biomarkers for improved therapy monitoring in EwS. Until now, response assessment is largely based on MRI and CT imaging, implying restrictions on closely repeated performance and limitations on the differentiation between vital tumor and reactive stromal tissue. Particularly in patients with prognostic unfavorable disseminated disease, ctDNA is a valuable addition for the assessment of therapy response. Clin Cancer Res; 22(17); 4356–65.

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Introduction

Ewing sarcoma (EwS) is an aggressive bone and soft-tissue tumor occurring from birth to late adulthood with an overall incidence of 1 case per 1 million. Half of all patients are diagnosed as adolescents; the median age at diagnosis is 15 years. Primary tumors are predominantly localized in the pelvis, chest wall, and long tubular bones of the extremities. Main sites of metastasis are lung, bone, and bone marrow (1). Around 25% of patients are diagnosed with disseminated disease at multiple sites. Their prognosis is particularly dismal with a 5-year overall survival between 10% and 30% depending on additional risk factors (2, 3).

EwS belongs to the group of small round blue cell tumors in childhood. Differentiation from other entities of this group has substantially benefited from comprehensive genetic and functional characterization, resulting in the availability of additional immunochemical markers and reliable molecular tests for detection of the underlying EWSR1 gene rearrangement. Validation of the characteristic rearrangement of EWSR1 and a member of the ETS family is now part of the standard diagnostic workup and classification of EwS. Most prevalent are the fusion genes EWSR1-FLI1 (85%–90%) and EWSR1-ERG (~10%); rare, but recurrent variants are also readily identifiable by RT-PCR (4–6).

Despite the broad spectrum of clinical presentation at diagnosis and the variable phenotypic differentiation, initial therapy follows a relatively uniform regimen of intense multiagent induction chemotherapy in current therapy trials. Assessment of therapy response during this intense treatment phase is largely based on follow-up MRI and CT imaging studies of primary lesions at selected time points. In addition, the value of functional imaging for staging and response assessment during induction therapy is currently addressed, e.g., in the ongoing EWING2008 trial by prospective evaluation of 18F-fluoro-deoxyglucose PET/CT. After initial multiagent chemotherapy, local therapy, i.e., surgical resection, irradiation, or combination of both, is mandatory for definitive disease control.

If tumor resection is feasible, histologic response, classified as percentage of vital tumor cells after completion of induction therapy, is used to stratify for the intensity of the following consolidation therapy. However, complete resection and quantification of tumor necrosis are often not possible because of two characteristic EwS features. First, due to the typical involvement of
Translational Relevance

Due to the lack of tumor-associated serum markers, therapy monitoring in Ewing sarcoma is essentially based on imaging procedures that do not allow stratification upon early chemotherapy response assessment. Prospective evaluation of alternative, functional imaging techniques is difficult to conduct in these young and often critically ill patients due to tedious procedures frequently requiring sedation and cumulative high radiation doses. Hence, all patients are currently treated with the same intense chemotherapy regimen during the first 5 months.

Using tumor-associated genomic fusion sequences as non-invasive biomarkers will allow for closer therapy monitoring and might help to identify patients at risk earlier during the course of therapy. This may facilitate improved risk adapted, personalized therapy. Furthermore, quantification of circulating tumor DNA in the follow-up period might help to detect a relapse on a molecular level. The approach described here is also applicable to any malignancy with recurrent chromosomal translocation.

Circulating Tumor DNA in Ewing Sarcoma

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Materials and Methods

Cell lines

Four EWSR1-FLI1-positive human EwS cell lines (A673, RD-ES, TC-71, and VH-64) were used for tumor generation in immunodeficient mice. TC-71 and RD-ES cells were obtained from the German Resource Center for Biologic Material (DSMZ); the A673 cell line was obtained from the American Type Culture Collection (ATCC); and the VH-64 cell line was provided by Frans van Valen in 2010 (18). Cell lines were authenticated by the cell line-specific genomic fusion sequence using single PCR directly before xenotransplantation (19). All cell lines were cultured in RPMI medium supplemented with 10% FBS, α-glutamine, and antibiotics at 37 °C in 5% CO2 on collagen-coated flasks. A673 and TC-71 cells express a fusion transcript consisting of EWSR1 exon 1-7 and FLI1 exon 6-9 representing EwS type 1, whereas VH-64 and RD-ES cells express a fusion transcript of EWSR1 exon 1-7 and FLI1 exon 5-9 representing EwS type 2 (20).

Xenograft mouse model

NMRI nu/nu mice and NOD.Cg-PkdCp+/+H2Begfp+/+Tcrd+/+ fed with 105 EWSR1-FLI1-positive cells to model localized disease or i.v. with 3 × 105 EWSR1-FLI1-positive cells to recapitulate disseminated EwS. Mice were monitored daily for tumor formation and symptoms of disease. EDTA-blood samples (100 μL) were taken once a week and centrifuged immediately at 600 g for 10 minutes for plasma recovery. At the end of the study, plasma was acquired from about 1 mL blood samples. Plasma samples were stored at −80 °C until DNA isolation.

Volumes of subcutaneous tumors were calculated from caliper measurements. Tumor burden in the i.v. injected, metastatic mouse model was evaluated postmortem by the number and size of metastases. EWSR1-FLI1 fusion gene-specific single PCR was used to confirm tumor tissue by the cell line-specific genomic EWSR1-FLI1 fusion sequence previously sequenced and deposited in the NCBI GenBank with accession numbers JX266518, JX266520, JX266523, and JX266525.
To visualize organ metastases ex vivo, resected samples were fixed with paraformaldehyde, embedded in agarose, and scanned on a preclinical ultra-high field MRI (7 Tesla, ClinScan; Bruker) using a T2-weighted spin-echo sequence (resolution: 50 × 50 × 200 μm). For histologic examinations, resected tumors and metastases were fixed with paraformaldehyde and embedded in paraffin. Sections (3 μm) were stained with hematoxylin and eosin and the antibodies CD99 (1:100 dilution; Dako clone D2E7) and Ki-67 (1:100 dilution; Dako clone MIB1).

**Patients and sample collection**

Twenty EwS patients (7 females and 13 males; median age, 14.6 years; range, 2–38 years) were accrued to our study. Nineteen individuals had a new diagnosis of EwS and were enrolled to the EWING2008 trial (NCT00987636). Induction chemotherapy consists of six cycles of vincristine, ifosfamide, doxorubicin, and etoposide (VIDE) in all patients followed by local therapy and consolidation therapy depending on individual risk stratification. One patient, UPN6, was included at the time of first relapse and treated in accordance with national guidelines. Histology and fusion transcript or FISH results were confirmed by a reference pathology review. Written informed consent was obtained from all patients or their legal guardians in accordance with the Declaration of Helsinki. Clinical and molecular parameters are summarized in Table 1.

For each patient, depending on the individual’s body weight, 2 to 9 mL EDTA blood samples were collected at the time of diagnosis and during the course of chemotherapeutic treatment. Research blood specimens were always drawn at the time of induction therapy, 40 during consolidation therapy and surveillance, 52 after relapse diagnosis). Plasma was separated from peripheral blood cells by centrifugation at 600 g for 15 minutes within 2 hours after blood was drawn and stored at –80°C. The EWING2008 protocol includes scheduled assessment of MRI or CT scan and optional PET/CT using a T2-weighted spin-echo sequence (resolution: 50 × 50 × 200 μm). For histologic examinations, resected tumors and metastases were fixed with paraformaldehyde and embedded in paraffin. Sections (3 μm) were stained with hematoxylin and eosin and the antibodies CD99 (1:100 dilution; Dako clone D2E7) and Ki-67 (1:100 dilution; Dako clone MIB1).

**Identification of the patient-specific genomic EWSR1-FLI1 or EWSR1-ERG fusion sequences from primary tumor samples**

DNA was isolated from fresh frozen cryopreserved biopsies or formalin-fixed paraffin-embedded tissues using the QIAamp DNA Blood and Tissue Mini Kit (Qiagen GmbH) according to the manufacturer’s instructions. Genomic EWSR1-FLI1 fusion sequences from cryopreserved tumor biopsies were identified by a nested multiplex long-range PCR (MLR-PCR) assay described earlier (19). EWSR1-ERG fusion sequences were detected by an analogous MLR-PCR assay, including a reverse ERG primer set (Supplementary Table S1). Formalin-fixed paraffin-embedded tissues are highly disadvantaged when using this original method. To adjust for the higher fragmentation of DNA isolated from formalin-fixed paraffin-embedded tissues, a larger amount of template DNA is required, and additional multiplex-PCR primers were designed for amplification of shorter PCR products (Supplementary Table S1). All PCRs were performed using the AccuPrime Taq DNA Polymerase System (Invitrogen) according to the manufacturer’s instructions. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced by Eurofins Genomics.

**Isolation and quantification of cell-free circulating DNA**

After thawing, plasma samples were centrifuged at 11,000 g for 3 minutes to remove residual cells or cell debris. Cell-free DNA was isolated using the QIAamp Circulating Nucleic Acid Kit (Qiagen). ctDNA was quantified by the detection of the cell line or patient-specific EWSR1-FLI1 or EWSR1-ERG fusion genes using breakpoint spanning primers and probe sets (Supplementary Table S2). Quantification was performed by droplet digital PCR (ddPCR) on a QX100 droplet generator and reader system (Bio-

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Abbreviations: FFPE, formalin-fixed paraffin-embedded tissue; FFT, fresh frozen tissue; n.a., not applicable, primary tumor resection.

*Referring to UCSC genebank built GRCh37/hg19.

**Table 1. Patient characteristics and genomic breakpoint positions**

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Rad). The ddPCR mixture was composed of 6 µl cell-free DNA, 900 nmol/L of each primer, 250 nmol/L probe, 2x ddPCR Supermix for Probes. The fluorescence signal after ddPCR was determined on the QX100 droplet reader and analyzed with Quanta Software (Version 1.5.38). Cases with less than three follow-up samples were quantified on a CFX96 real-time PCR detection system with EvaGreen Supermix (Bio-Rad). To calculate the absolute number of EWSR1-FLI1 or EWSR1-ERG, the fusion-specific probe signal was normalized to a signal of the single copy genes mouse β-actin or human albumin and plasma volume used for DNA extraction, respectively. The sensitivity of the ddPCR assay for the detection of ctDNA copies in plasma was about 0.1%.

**Results**

**Quantification of genomic EWSR1-FLI1 fusion sequences in the plasma of EwS xenograft mice**

To evaluate whether tumor-specific genomic fusion sites are quantifiable in small plasma samples and can be used as molecular serum markers for tumor growth monitoring in children, we first examined ctDNA levels in two EwS xenograft mouse models. Tumor size, tumor vitality, and the number of metastatic sites can be studied postmortem in mice, facilitating evaluation of the correlation between ctDNA levels and tumor burden. Furthermore, the genomic EWSR1-FLI1 fusion sequence specific for each injected cell line is already known from earlier work (19).

Four different EwS cell lines injected in NMRI nu/nu mice and in vivo mice were analyzed in our preclinical study to estimate cell line and recipient-related effects of the ctDNA level in mouse plasma.

The tumor engraftment was different in NSG and NMRI nu/nu xenograft mouse models. After s.c. or i.v. injection, all NSG mice developed tumors independent of EwS cell line injected, at which VH-64 and RD-ES cells, representing EwS type 2, showed a decelerated tumor engraftment (Supplementary Fig. S1A and S1B). After i.v. injection, tumors were preferentially located in the liver, kidney, and lung (Supplementary Fig. S1C).

In contrast, NMRI nu/nu mice demonstrated a slower and overall reduced tumor engraftment. Nearly all NMRI nu/nu mice developed tumors after s.c. injection of A673 and TC-71 cells, but no tumors were palpable after s.c. injection of VH-64 and RD-ES cells (Supplementary Fig. S1A). After i.v. injection of A673 and TC-71 cells, 58% of mice (14/24) presented with metastases, only 20% of mice (2/10) developed metastases after i.v. injection of VH-64 cells, and no tumor growth was observed after i.v. injection of RD-ES cells (Supplementary Fig. S1B). Metastases developed in lung and bone similar to the metastatic pattern commonly observed in EwS patients; no additional organs were affected (Supplementary Fig. S1C).

Immunohistochemical studies confirmed tumor tissues as EwS by strong membranous CD99 staining in both mouse models with highly active proliferation as more than 60% of tumor cells had a positive Ki-67 signal (Supplementary Fig. S1C).

Plasma of xenograft mice was monitored weekly for the presence of the tumor-specific fusion sequence. We could not detect any EWSR1-FLI1 copies in the plasma of s.c.-injected mice during the time of tumor growth. A positive EWSR1-FLI1 signal was detectable in 70% (14/20) of mice only at the end of the study when the localized tumor reached the maximum size of around 1,000 mm³ (Fig. 1A). In median, 110 copies (range, 15–360) EWSR1-FLI1 per 1 mL plasma compared with 1,700 copies (range, 800–9,400) mouse-albumin per 1 mL plasma were detectable.

In contrast, i.v.-injected mice, modeling metastatic disease, showed positive EWSR1-FLI1 signals in median 8 days before first symptoms of disease became visible (Fig. 1A). ctDNA copies were detectable in all transplanted mice. Monitoring of the ctDNA level during the time of tumor development revealed an increase of EWSR1-FLI1 copies with tumor progression (Fig. 1B). Mice with i.v.-injected VH64 or A673 cell were classified in minor, medium, and heavy infiltration based on the number of metastases observed.

The xenograft mouse models confirmed the reproducible detection of cell line-specific EWSR1-FLI1 copies in correlation with the overall tumor burden from very small blood samples. We therefore continued to evaluate the clinical applicability of genomic fusion sites as molecular plasma marker in children and young adults treated for EwS.

**Quantification of genomic fusion sequences in plasma samples of EwS patients**

Quantification of EWSR1-FLI1 or EWSR1-ERG fusion sites in the patient’s plasma requires the identification of the individual genomic fusion sequence as a first step. In our present study cohort, we identified 19 patients with a EWSR1-FLI1 fusion gene and 1 patient with a EWSR1-ERG fusion sequence (UPN6). In 12 EWSR1-FLI1-positive patients, the rearrangement resulted in the fusion of EWSR1 exon 7 to FLI1 exon 6, generating the most frequent type 1 fusion transcript (21). Four patients (UPN3, 4, 11, and 17) expressed the fusion transcript EWSR1 exon 1-7 fused to FLI1 exon 5-9, representing the second most frequent EwS fusion type 2. Three patients showed infrequent fusion transcripts; EWSR1 exon 7 fused to FLI1 exon 8 (UPN10 and 14) and EWSR1 exon 9 fused to FLI1 exon 8 (UPN5), respectively. The underlying individual genomic fusion sequences are shown in Supplementary Fig. S2.

The patient-specific genomic EWSR1-FLI1 and EWSR1-ERG fusion sequences were used as templates for the design of fusion gene–specific primer probe sets. We first analyzed plasma samples at the time of initial diagnosis and relapse expecting that these samples are most likely positive for ctDNA detection. Fourteen patients had plasma samples at initial diagnosis, 2 patients had plasma samples at initial diagnosis and relapse, and 1 patient had two plasma samples at two different relapse times available. We were able to detect ctDNA copies in 18 of 20 plasma samples and observed a consistent correlation between tumor volume and ctDNA copy number (Fig. 2). Copy numbers of EWSR1-FLI1 or EWSR1-ERG normalized to the copy number of the single copy gene albumin varied between 0.003 and 0.56. Two patients (UPN7 and 10) tested negative for EWSR1-FLI1 copies. Both patients had small localized tumors (3.2 mL in the popliteal fossa and 14 mL in the neck, respectively) that were resected before first plasma samples had been taken.

To evaluate the use of genomic fusion sequences as serum markers for therapy monitoring, we further analyzed plasma samples during the course of chemotherapeutic treatment. A total of 213 (median 7) follow-up samples were collected in 17 patients. A summary of detectability of ctDNA copies and tumor volume during the initial chemotherapy (VIDE1 – VIDE6) is shown in Fig. 3.

Two patients with initially resected tumors (UPN7 and 10) had no detectable ctDNA copies in all follow-up samples analyzed.
confirming the high specificity of PCR-based quantification of genomic rearrangement; no false-positive results were generated in the absence of specific template DNA. Fast reduction of the ctDNA copy number after onset of chemotherapy could be observed in most cases without initial tumor resection. At the beginning of the second VIDE block, 60% of the patients (9/15) have no or only few EWSR1 fusion sites detectable. Of the remaining 6 patients, 3 patients were ctDNA negative before VIDE block three. All of these 12 patients had a good therapy response, and imaging confirmed significant tumor reduction (exemplarily see Fig. 4 UPN1 and 20).

Three patients demonstrated a different ctDNA response pattern (Fig. 4). In patient UPN19 who had a disseminated tumor with primary infiltration of the os ilium, single EWSR1-FLI1 copies were still detectable at the third VIDE block. This patient relapsed about 10 months after the initial diagnosis (7.5 mL metastasis in the humerus) that could be discovered by an increase of ctDNA copies in the patient's plasma. In the following course of disease, the patient developed a local relapse in the os ilium and multiple lung metastases that could always be detected by an increase of the ctDNA plasma levels (Fig. 5). In patient UPN5, ctDNA levels were detectable during the entire induction chemotherapy (VIDE1–VIDE6). This patient also relapsed 3 years after initial diagnosis. Despite the very small size of the lymph node metastasis (0.8 mL), an increase at the ctDNA level could be detected at that time.

Patient UPN6 was included in our study at the time of first relapse. This patient initially showed a fast decrease of ctDNA copy numbers, but 3 months later had a recurrence of EWSR1-ERG fusion sequences in the plasma, indicated by the small peak in the graph (Fig. 4). The patient developed a relapse 14 months after treatment began. The relapse site at the second cervical vertebra was difficult to identify by imaging studies, whereas increasing EWSR1-ERG plasma DNA indicated the disease reoccurrence.

Our present results show that the copy number of genomic fusion DNA fragments in the plasma of EwS patients correlates with tumor burden and may indicate relapse development.
Discussion

We investigated the utility of the genomic EWSR1 fusion sequence as a plasma marker in children and adolescents with EwS. ctDNA, small DNA fragments released from the tumor tissue to the blood stream by various mechanisms, has been shown to be representative of the tumor genome of many cancer types and constitutes a valuable additional source of tumor material, particularly when incision biopsies are difficult to obtain from critically located tumors (22). The term Liquid Biopsy has been coined to this approach and pictures the potential of cell-free DNA sampling with minimal invasiveness, enabling repeated sampling at multiple times during treatment, and the possibility of real-time monitoring.

Given the incidence and spectrum of malignant tumors in humans, applications of ctDNA for the genetic assessment of solid tumors and monitoring during therapy have almost exclusively been explored in adult patients with epithelial tumors. Initial studies in subjects with colorectal cancer undergoing surgery or chemotherapy found that ctDNA measurements could be used to monitor tumor dynamics. The ctDNA appeared to be a more reliable and sensitive indicator than the current standard biomarker (CEA) in this cohort of subjects (10). Dawson and colleagues further showed a greater dynamic range, greater correlation with changes in tumor burden, and earlier measure of treatment response of ctDNA quantification compared with CA 15-3 or circulating tumor cells, following the clinical course of 20 patients with metastatic breast cancer (8). Meanwhile, many additional tumor types have been examined for their representation in plasma samples, including non–small cell lung cancer, gastroesophageal, hepatocellular, pancreatic, bladder, prostate, ovary, melanoma, head, and neck cancers (23–25).

These cancer types share commonly acquired key driver gene mutations, e.g., PIK3CA, TP53, EGFR, APC, KRAS, and NRAS. The limited number of recurrent single-nucleotide changes at hot-spot mutation sites enabled the development of ready-made assays (23–26).
However, pediatric malignancies vary significantly from adult cancer with respect to incidence and typical tumor types and therefore also exhibit an entirely different spectrum of cancer-associated mutations. A large proportion of entities are specified by the presence of recurrent fusion genes as a result of chromosomal translocations. This is a common characteristic of tumors of mesenchymal origin, but also increasingly identified in current tumor sequencing data from epithelial neoplasia (27).

Both aspects, the lack of common hot-spot mutations and the presence of distinct translocations, are particularly applicable to EwS. Three recent studies consistently described the genomic landscape of EwS as relatively silent (13–15). In an analysis of 27 cancer types of all age groups, EwS revealed the second lowest median frequency of nonsynonymous mutations, 10- to 100-fold below the frequency observed in other entities studied, e.g., colorectal cancer, lung cancer, or melanoma (28). Contrary, the presence of one of the characteristic fusion genes involving EWSR1 and a partner gene of the ETS family is highly prevalent and therefore became a diagnostic criterion in current guidelines (29).

Application of chromosomal translocations as biomarkers for tumor cells has several general advantages and disadvantages compared with hot-spot single-nucleotide mutations more commonly applied for ctDNA quantification.

Due to the combination of chromosomal material from different chromosomes, the resulting fusion sequence is absolutely unique and has a particularly distinctive nucleotide composition, conferring high sensitivity and specificity to probe sets spanning the chromosomal breakpoint. Base calling in next-generation sequencing algorithms is less prone to false-negative and false-positive calls given the extended stretches of nucleotides differing from wild-type reference sequence. Because the underlying characteristic fusion gene is not only cancer associated but causative and therefore stable during disease development, intratumor heterogeneity and clonal selection under treatment are not a critical issue in contrast to emerging clonal heterogeneity under targeted therapy, e.g., with EGFR antibodies (30, 31).

However, each genomic fusion site is an individual sequence within large intronic gene segments requiring specific approaches to facilitate detection and sequencing (19). The patient-specific genomic fusion site in our study has been identified from biopsy material used for the histologic diagnosis. In principle, identification of the fusion sequence is also feasible from plasma DNA using appropriate sequence library enrichment strategies and next-generation sequencing techniques (23, 32).

Stated previously, EwS is an overall rare disease in comparison with cancer types with high prevalence of hot-spot mutations studied for their utility as plasma biomarkers. We were uncertain at the beginning of the study, whether ctDNA assays would be sufficiently sensitive to detect tumor kinetics in EwS and wanted to confirm the technical feasibility, in particular dealing with small sample volumes, before initiation of repeated blood sampling from children. We therefore decided to conduct an initial proof-of-concept study in preclinical EwS xenotransplant mouse model. Despite very small plasma volumes available, tumor-specific genomic fusion sequences were detectable in xenotransplanted mice in relation to disease extent. Comparison of the ctDNA dynamic between subcutaneous and disseminated mouse model supports the assumption that not only the tumor size, but also the localization, respectively the access to blood circulation, is a determinant of ctDNA copy number. These results indicate that ctDNA can also be used in preclinical mouse models as plasma tumor marker of EwS without the need to sacrifice animals at several time points to assess disease development or therapy response.

In clinical care of patients with EwS, there are two challenges that could benefit from additional information on therapy response as determined by ctDNA. One aspect is the difficulty of radiographic assessment on the distinction between residual...
tumor tissue, necrotic neoplastic cells, and reactive inflammatory and stromal cells. In several of our cases, the discrepancy between tumor volumes, calculated from imaging, and the detection of ctDNA was attributable to the lack of definition of clear-cut tumor margins after onset of induction therapy. The second limitation of the current stratification is the fact that not all cases can undergo surgery for local disease control due to unfavorable or disseminated tumor localization. Stratification based on the percentage of vital tumor cells after completion of induction therapy is therefore not possible and relies on pretreatment parameters in those cases, ignoring the actual response on multiagent chemotherapy received until then.

DNA sequences specific for the primary tumor can be isolated from blood samples either from circulating tumor cells or from circulating cell-free DNA. When circulating tumor cells were directly compared with tumor-specific cell-free DNA from the same blood sample in larger cohorts of patients with diffuse large B-cell lymphoma, quantitatively more tumor DNA was found in the plasma (33, 34). Likewise, disease detection from plasma was more sensitive at the time points of overt disease, especially at relapse, compared with the cellular blood fraction, although this disease has higher numbers of circulating tumor cells than sarcomas. Furthermore, measurement of disease from ctDNA, but not from circulating tumor cells, was significantly correlated with tumor volume as measured by $^{18}$FDG PET/CT scan (33).

Circulating tumor cells in peripheral blood samples have been found qualitatively present in only one fifth of EWS patients at diagnosis, most frequently in cases with large tumors (24), whereas ctDNA in our study was detectable in all patients before treatment start, except two cases (UPN7 and 10) with small extraosseous tumors, resected before first blood samples were taken. The ctDNA is the main source of tumor-associated DNA in our study, since sample management ensured immediate centrifugation and therefore separation of cellular fraction. Furthermore, disintegration of circulating tumor cells by sample handling and centrifugation is unlikely to contribute significantly to the copy number of EWSR1-FLI1– or EWSR1-ERG–specific fusion fragments because the potential number of circulating tumor cells in the blood sample is negligible compared with ctDNA fragments, given the average copy number at diagnosis.

The number of plasma ctDNA copies decreased with tumor regression and increased in case of relapse development. No false-positive ctDNA copies were detected in any of the samples tested at any time point. Relatively high ctDNA copy numbers were recorded when metastases occurred at previously unaffected sites. A possible explanation is that micrometastatic lesions smaller...
than a few millimeters are not detectable by PET/CT or MRI scans, but in aggregate may make a large contribution to the total tumor burden (10).

Whether the ctDNA copy number is capable to differentiate localized metastases (UPN 5) from developing disseminated metastatic disease (UPN6 and 19) has to be subject of larger prospective studies, investigating ctDNA in combination with established clinical prognosis parameters. However, besides the potential to monitor the disease burden during induction therapy in order to identify patients at high risk of treatment failure, follow-up samples during and after maintenance therapy may also prove instrumental in early detection of disease recurrence. At present, EwS relapse is an incurable disease (35, 36). A further and careful prospective validation of early detection of molecular relapse is required. If such detection is feasible and would allow starting therapeutic intervention at an early stage, this might lead to an improved survival after EwS relapse.

The availability of a serum marker for disease monitoring in children is particularly beneficial. Its implementation will alleviate exposure to radiation, the need of sedation for performance of radiographic studies in young children, and the constraints on frequently therapy monitoring.

In the present study, we evaluated for the first time the use of a genomic fusion sequence as stable, noninvasive tumor biomarker in EwS. ctDNA containing the characteristic and causative EWSR1-FLI1 and EWSR1-ERG rearrangements proved a suitable marker for monitoring tumor burden at diagnosis, response to therapy, and disease relapse, complementary to regular imaging surveillance. The analogous approach is applicable to any translocation-positive malignancy in both pediatric and adult oncology.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


Correction: Genomic EWSR1 Fusion Sequence as Highly Sensitive and Dynamic Plasma Tumor Marker in Ewing Sarcoma

In this article (Clin Cancer Res 2016;22:4356–65), which was published in the September 1, 2016, issue of Clinical Cancer Research (1), EURO EWING Consortium funding was incorrectly listed in the grant support statement. The full statement should read as follows: “This research was supported by grants of the Madeleine Schickedanz Kinderkrebs-Stiftung, Schornsteinfeger helfen krebskranken Kindern, German Cancer Aid to UD (DKH 108128) and the EraNet consortium PrOspectiveVAlidationofBiomarkersinEwingSarcoma (PROVABES ERA-Net-TRANSCAN (01KT1310)). This project has received funding from the European Union’s Seventh Framework Programme for research, technological development and demonstration under grant agreement no 602856 (to M. Metzler and U. Dirksen).” The authors regret this error.

Reference

Published online January 16, 2017.
doi: 10.1158/1078-0432.CCR-16-2685
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Genomic $EWSR1$ Fusion Sequence as Highly Sensitive and Dynamic Plasma Tumor Marker in Ewing Sarcoma

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