9 Using Liquid Biopsy in the Treatment of Patient with OS

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Abstract

Liquid biopsies encompass a number of new technologies designed to derive tumor data through the minimally invasive sampling of an accessible body fluid. These technologies remain early in their clinical development, and applications for patients with osteosarcoma are actively under investigation. In this chapter, we outline the current state of liquid biopsy technologies as they apply to cancer generally and osteosarcoma specifically, focusing on assays that detect and profile circulating tumor DNA (ctDNA), microRNAs (miRNA), and circulating tumor cells (CTCs). At present, ctDNA assays are the most mature, with multiple assays demonstrating the feasibility of detecting and quantifying ctDNA from blood samples of patients with osteosarcoma. Initial studies show that ctDNA can be detected in the majority of patients with osteosarcoma and that the detection and level of ctDNA correlates with a worse prognosis. Profiling of ctDNA can also identify specific somatic events that may have prognostic relevance, such as 8q gain in osteosarcoma. miRNAs are stable RNAs that regulate gene expression and are known to be dysregulated in cancer, and patterns of miRNA expression have been evaluated in multiple studies of patients with osteosarcoma. While studies have identified differential expression of many miRNAs in osteosarcomas compared to healthy controls, a consensus set of prognostic miRNAs has yet to be definitively validated. Recent studies have also demonstrated the feasibility of capturing CTCs in patients with osteosarcoma. The development of assays that quantify and profile CTCs for use as prognostic biomarkers or tools for biologic discovery is still in development. However, CTC technology holds incredible promise given the potential to perform multi-omic approaches in single cancer cells to understand osteosarcoma heterogeneity and tumor evolution. The next step required to move liquid biopsy technologies closer to helping patients will be wide-scale collection of patient samples from large prospective studies.

Keywords

Liquid biopsy · Circulating tumor DNA · MicroRNA · Circulating tumor cells · Prognosis · Diagnosis · Biomarker · Surveillance · Tumor evolution · Sarcoma · Osteosarcoma

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The Emerging Field of Liquid Biopsy in Cancer

"Liquid biopsies" hold incredible promise to transform the way we treat patients with osteosarcoma. Liquid biopsy describes an array of assays designed to extract tumor information from body fluid, including peripheral blood, cerebral spinal fluid, urine, or effusions, that may be more easily accessible than tissue from a surgical biopsy. These technologies provide a noninvasive opportunity to study cancer biology and derive clinically useful information at numerous times during a patient's treatment for cancer. Specific advancements include a more comprehensive understanding of disease biology, a means of risk stratification, a method to measure treatment response, a tool for early identification of relapses, and a means to identify mechanisms of treatment resistance. While numerous studies now show that tumor material can be detected in the blood of patients with cancer, tumor-derived nucleotides, tumor cells, and cell fragments remain a very small fraction of the components of the blood, even in cancer patients with a high burden of disease. Therefore, the major challenge to adapting liquid biopsy technologies to each cancer type is the identification of disease hallmarks that distinguish cancer material from the patient's normal blood components. As this field of cancer biology rapidly grows, studies describing approaches for the identification of circulating tumor material in patients with osteosarcoma are just beginning to emerge. In this chapter, we aim to provide an overview of the current state of liquid biopsy across oncology, within pediatric oncology, and the nascent work that has been done to develop liquid biopsy assays for patients with osteosarcoma. Finally, we will discuss future directions for these assays and how they may ultimately improve outcomes for patients with osteosarcoma.

The first descriptions of freely circulating DNA in the peripheral blood came about in 1948 [1]. The first description of circulating DNA in patients with cancer occurred in the 1970s and 1980s [2, 3]. Since that time, assays to detect and characterize circulating tumor (ctDNA) have improved enormously through advancements in PCR and next-generation sequencing, which have become the most prevalent means of performing liquid biopsies [4–6]. Although there is a growing literature evaluating liquid biopsy in cancer, relatively few studies have demonstrated clinical utility or validity of these assays [5]. To date, two ctDNA assays have gained FDA approval for use in adult cancers [7, 8]. Nevertheless, an increasing number of studies have demonstrated early evidence for the use of ctDNA for disease diagnosis, prognostication, measurement of residual disease, identification of genomic alterations for targeted therapy, and exploration of disease biology.

More recently, numerous alternative methods of ascertaining information about a tumor through liquid biopsy have been developed including analysis of circulating RNA (primarily microRNA (miRNA)), circulating tumor cells (CTCs), extracellular vesicles (EVs), exosomes, tumor educated platelets (TEPs), proteins, and metabolites [9]. These areas of exploration all harbor an opportunity to advance the care we provide for patients with osteosarcoma in different ways. For the purposes of this chapter, we will focus on liquid biopsy strategies which have been applied in some way to osteosarcoma, which include the detection and profiling of ctDNA, CTCs, and miRNA.

Liquid Biopsy Technologies and Their Adaptation to Osteosarcoma

While ctDNA has been evaluated in many diseases as a type of liquid biopsy, conventional methods of analysis relied upon detection of recurrent hotspot mutations in genes such as *KRAS* and *EGFR*, which are common in carcinomas, but rare in sarcomas [7, 10–13]. Instead, many sarcomas harbor genomes with characteristic translocations or copy-number changes. Sarcomas require approaches to ctDNA detection and quantification that are tailored to the recurrent genomic aberrations found in these diseases as well as the particular clinical context in which

the assay will be used. Similarly, approaches to detection of CTCs require that such assays leverage characteristic features of the sarcoma tumor cell. Evaluation of miRNAs may be performed using similar techniques to those described in other cancers, but profiling results must be analyzed to identify the specific miRNAs secreted by osteosarcoma tumors. Here we describe ways in which knowledge of osteosarcoma biology may be leveraged to harness ctDNA, CTC, and miRNA assays as a means of liquid biopsy for patients with osteosarcoma.

ctDNA

The detection of ctDNA relies on the identification of somatic variants that distinguish tumor DNA from germline DNA. Several studies have shown that pediatric solid tumors harbor few recurrent single-nucleotide variants, diminishing the value of hotspot focused ctDNA assays for these diseases [14–16]. In osteosarcoma, one group demonstrated that next-generation targeted sequencing of a panel of genes designed to detect a combination of recurrent single-nucleotide variants (SNVs) and focal structural variants was able to identify ctDNA in six of eight cases of osteosarcoma [17]. However, genomic studies of the most common pediatric solid tumors would suggest that a reasonably sized panel of genes targeting only SNVs would be able to detect ctDNA in only a subset of patients [18–28].

Pediatric solid tumors are typically characterized by structural variants, including recurrent translocations and frequent copy-number alterations [14]. For tumors characterized by recurrent copy-number changes, such as osteosarcoma, whole-genome or whole-exome sequencing can be utilized to detect and quantify ctDNA [6]. Recent studies have utilized ultralow-pass whole-genome sequencing (ULP-WGS), with genome coverage as low as 0.1-1x, to identify ctDNA in diseases with genomes characterized by widespread structural events by employing computer algorithms such as the iChorCNA to use segmental and chromosomal alterations to estimate the ctDNA content of a sample [29]. This approach lies in contrast to next-generation sequencing strategies utilized for detection of ctDNA in translocation positive sarcomas, where intronic regions that typically host recurrent rearrangements are enriched for deep sequencing [30].

In osteosarcoma, landscape sequencing studies have shown that these tumors host few recurrent SNVs, have one of the most complex genomes in cancer, and frequently contain aneuploidy and chromothripsis [20, 27]. Unlike other types of pediatric solid tumors, copy-number and translocation events appear to be nearly stochastic, increasing the challenge of bringing a low-cost sequencing technology to the identification of ctDNA in the blood. In recent work, ULP-WGS was used to effectively detect ctDNA in patients with localized, metastatic, and recurrent osteosarcoma [30]. While this approach has limitations in terms of sensitivity for ctDNA, this technique is well adapted to the osteosarcoma genome. As ctDNA assays become more adaptive, it may be possible that unique CNAs and rearrangements harbored within each individual's tumor may provide an opportunity to develop patient-specific ctDNA assays. However, such an approach has yet to be described for patients with osteosarcoma.

Another unique hallmark of the cancer genome is the methylation pattern of DNA. Numerous studies have demonstrated that different cancer types harbor unique methylation patterns that can distinguish each cancer from normal tissues and other cancer types. Recent studies have demonstrated the feasibility of utilizing methylation profiling to categorize small round blue cell tumors, such as Ewing sarcoma, osteosarcoma, desmoplastic small round cell sarcoma, and synovial sarcoma [31]. Similar methylation profiling has been applied to sequencing methylomes in cell-free DNA demonstrating a similar ability to detect ctDNA and differentiate cancer types based on methylation profiles [32, 33]. While this has been accomplished for osteosarcoma using tissue sequencing, it has not been performed using ctDNA [31]. This may ultimately prove to be a sensitive means of early detection in patients at risk of sarcomas, improving diagnosis in situations in which diagnostic

tissue is not attainable and improving disease surveillance.

Circulating Tumor Cells

Circulating tumor cells (CTCs) are intact tumor cells found in the bloodstream as single cells or clusters and have been postulated to exist since it was first understood that tumors could metastasize to other locations in the body. The term CTC generally refers to tumor cells derived from solid tumors that do not otherwise circulate in the blood, as opposed to malignancies of the blood. CTCs may be viable or apoptotic at the time of analysis, with viable CTCs likely representing tumor cells with the potential to form metastases [34]. CTCs are typically isolated through positive detection using markers on the surface of the tumors cells or physical cell characteristics such as size, electrical charge, density or deformability or through negative detection by removing noncancerous cells from a blood sample. Traditionally, analysis of CTCs focused simply on detection and enumeration of CTCs; however, advances in single-cell analysis have opened the door to a wide range of studies, including single-cell sequencing, epigenome analysis, and protein profiling [35, 36]. More recent studies have demonstrated the utility of a combination approach using CTC enrichment and RNA sequencing [37].

CTCs can now be reliably detected in patients with carcinomas using endothelial surface markers, primarily EpCAM [38]. Similar attempts to identify sarcoma cells have utilized surface markers, such as CD99 in Ewing sarcoma using flow cytometry [39, 40]. Vimentin has been shown to be a more ubiquitously expressed surface marker on sarcoma cells; [41] however, both vimentin and CD99 lack specificity with baseline expression of both markers on other circulating non-tumor cells. GD2 is another potential surface marker for isolating osteosarcoma CTCs [42, 43]; however, further work must be done to define solid tumor- and osteosarcoma-specific surface markers for CTC isolation. More recent attempts have utilized size selection for detection and isolation of CTCs in sarcomas and successfully isolated CTCs from patients with osteosarcoma [44].

miRNA

MicroRNAs are small (approximately 22 nucleotides in length) double-stranded RNAs that are thought to regulate gene transcription at the cellular level [45]. MicroRNAs were first characterized in the 2000s and, due to their relative stability, can be found ubiquitously in a variety of bodily fluids [46, 47]. These small double-stranded RNA sequences are produced in normal cells, and their expression is thought to be dysregulated in the cancer cell with the potential to act as oncogenic regulators of gene expression. miRNAs can be analyzed using RNA sequencing (RNA-Seq), quantitative PCR (qPCR), or microarrays and are being evaluated for a range of applications including early detection of cancer, diagnosis of cancer, and prognostication. While there is now a large literature evaluating these RNA profiles, little is known about the role in cellular regulation and the packaging of these molecules in the cytoplasm and extracellular space. It is believed that they are typically transported in extracellular vesicles, apoptotic bodies, high-density lipoprotein structures, and complexes with Argonaute proteins [47, 48]. As a biomarker, miRNAs are typically analyzed as a miRNA profile, consisting of a number of specific miRNAs, and the relative frequencies of each miRNA are analyzed as profiles relative to normal controls. These profiles may be developed through unbiased genome-wide profiling of miRNAs or by preselecting miRNAs for evaluation. These profiles may be used for early detection, diagnosis, or prognostication. Given that miRNAs have a role in regulating transcription, they may also eventually inform our understanding of disease biology.

The most extensive clinical studies of miRNA have evaluated miRNA profiles in patients at high risk of lung cancer. Two large studies have demonstrated that miRNA profiling may be able to augment low-dose CT screening in identifying

high-risk patients in need of a biopsy [49, 50]. miRNAs have been characterized in patients with osteosarcoma [51], and a number of studies have attempted to identify miRNA profiles associated with high-risk disease; however at this time, these studies have shown contradictory results, and larger studies with test and validation cohorts are needed.

Liquid Biopsy Applications in Cancer and Osteosarcoma

Early Detection and Diagnosis

The ability to detect genetic hallmarks of cancer in liquid biopsies has engendered optimism that these technologies may be used to augment traditional biopsies for cancer diagnosis. Such diagnostic liquid biopsies may be particularly beneficial in instances where viable tissue is difficult to obtain or the quality or quantity of a biopsy is not sufficient to arrive at a definitive diagnosis. For patients at an elevated risk of developing a malignancy, liquid biopsies may be a way to augment cancer screening regimens designed to detect cancer early, when tumors are expected to be more amenable to treatment.

Multiple lines of evidence suggest that ctDNA may be detectable at the time of diagnosis and prior to diagnosis in patients with osteosarcoma. While no published studies have detected prediagnostic ctDNA in patients who are later proven to have osteosarcoma, a previous study of a cohort of 72 patients with localized osteosarcoma with available banked plasma demonstrated that ctDNA was detectable using an ultralow passage whole-genome sequencing assay in 57% of newly diagnosed patients without prior knowledge of the tumor genome [52]. New means of collecting and isolating ctDNA and enhanced analytic algorithms are expected to increase the sensitivity of such assays for detection of osteosarcoma ctDNA.

While the majority of cases of osteosarcoma are thought to be sporadic, cancer predisposition syndromes, including Li–Fraumeni syndrome, and environmental exposures, such as prior treatment with radiation or chemotherapy, are known to increase the risk of developing osteosarcoma [53]. Although there are no published studies of liquid biopsies detecting occult osteosarcoma, there now exist multiple case reports of ctDNA being detected in women with no known existing tumor undergoing cell-free DNA prenatal testing, who were subsequently found to have cancer [54, 55]. One recent study has shown that using patient-specific NGS panels, ctDNA can be detected in patients with osteosarcoma and no radiologic detectable disease, speaking to the potential sensitivity of this assay in osteosarcoma [17]. These studies suggest that ctDNA assays may be adapted for early detection in cancer patients with an increased risk of developing malignancies, including osteosarcoma. Efforts are underway to improve the sensitivity of ctDNA assays which would be expected to improve the utility of these tests for early-cancer detection.

While the initial studies of liquid biopsies in osteosarcoma have focused on identifying patterns of DNA mutations, new studies in cancer now demonstrate that somatic methylation changes can be utilized to discriminate tumor DNA from germline DNA. This approach has the added benefit of being able to predict the type of tumor present in the patient when ctDNA can be detected by methylation patterns [32, 33]. CTCs may also provide diagnostic information, but studies to demonstrate the feasibility of such an approach remain aspirational. Multiple studies have also suggested that miRNA may be useful in discriminating the presence of osteosarcoma in patients compared to healthy controls [56, 57]; however, no specific miRNAs have shown promise across multiple studies, and it remains to be seen whether these biomarkers will be useful for diagnosing osteosarcoma.

Improving Risk Stratification of Newly Diagnosed Patients

Risk stratification of patients at the time of diagnosis remains an ongoing challenge in the clinical care of patients with osteosarcoma. The only existing strong prognostic factors for poor

outcomes for patients with high-grade disease remain the presence of metastatic disease and having an axial primary tumor [58, 59]. Further, for some patients, the presence of metastatic disease may be ambiguous if there are small pulmonary nodules of unclear significance. Conceptually, liquid biopsy may correlate with disease burden, or be associated with the presence of micrometastatic disease, and may provide an excellent biomarker for identification of high-risk patients, especially in instances where the presence of metastatic disease is not clear. Prognostication using liquid biopsy can be achieved through multiple approaches, including quantification of ctDNA or CTCs, or identification of high-risk genomic features such as Myc overexpression, or high-risk miRNA profiles.

Multiple studies have now demonstrated correlations between ctDNA quantification and stage and tumor size, although primarily in adult carcinomas [60–62]. Not surprisingly, early studies subsequently showed that ctDNA detection was associated with poor outcome. In one early study of patients with colorectal cancer, patients with detectable ctDNA had a 2-year overall survival of 48% compared to 100% for those without detectable ctDNA [63]. While there has not yet been a study attempting to correlate tumor size with ctDNA levels in osteosarcoma, we have demonstrated that binary ctDNA detection and increasing ctDNA levels are associated with event-free survival and overall survival in patients with localized osteosarcoma [52]. Although no ctDNA studies have shown that genomic features identified in ctDNA were associated with poor outcome, multiple genomic features in osteosarcoma identified in tumor tissue have been demonstrated to correlate with a poor outcome [26]. 8q gain was readily detectable in 74% of patients with detectable ctDNA in patients with localized osteosarcoma.

MicroRNAs have also been evaluated as potential prognostic markers in patients with osteosarcoma. A number of miRNAs have been evaluated, including miR-21, miR-106a miR-199a-3p, miR-143, miR-221, and miR-34b, however with varying results, sometimes upregulated and sometimes downregulated [64–70]. While these miRNAs seem to be detectable in the peripheral blood of patients with osteosarcoma, further work is needed to elucidate which miRNAs are consistently dysregulated and hold the potential for useful diagnostic and prognostic biomarkers.

Monitoring Response to Therapy and Detecting Relapse

Utilization of liquid biopsy for disease monitoring and surveillance holds particular promise in osteosarcoma given the challenges of using traditional imaging to gauge response to treatment [71] and the significant radiation exposure from CT scans during surveillance [72]. Further, markers of minimal residual disease (MRD), which have profoundly impacted the treatment of hematologic malignancies, are lacking in solid tumors.

All three analytes mentioned in this chapter hold the potential to improve disease monitoring and surveillance in osteosarcoma. To use ctDNA for disease monitoring, assays capable of quantifying ctDNA must be utilized. The iChor algorithm which discriminates ctDNA from genomic cfDNA by identifying copy-number variations is validated down to 3% ctDNA [29]. While it is not known whether this exceeds the threshold of radiologic detection for patients with osteosarcoma, it is likely that more sensitive assays will be required for ctDNA to be useful for disease monitoring and MRD detection. Nevertheless, case reports have demonstrated that ctDNA levels change following the treatment of osteosarcoma [30, 73]. To increase sensitivity of these assays, a number of strategies could be employed, including using patient-specific panels of copy-number changes or SNVs either using NGS or PCR-based assays [74]. Conversely, machine-learning techniques that differentiate tumor DNA from germline DNA are gaining increasing use and would likely prove useful for patients with osteosarcoma given the degree of copy-number changes seen in the osteosarcoma genome.

Similarly, CTCs and miRNA may prove useful for monitoring patients with osteosarcoma. Both CTC levels and miRNA levels are known to

change over time in patients with osteosarcoma [44, 67, 68]. However, the threshold for detection of these analytes even with the current technology is not well understood.

Understanding Tumor Heterogeneity and Evolution

Osteosarcoma harbors an extremely complex genome with yet unanswered questions about driving genomic alterations that may be further elucidated through deep sequencing of serial liquid biopsy samples [23, 25, 26, 75, 76]. Understanding spatial heterogeneity in osteosarcoma, like many solid tumors, has been hampered by sampling error of conventional biopsies, especially in patients with metastatic disease. Temporal heterogeneity, or how the osteosarcoma genome changes over time, has also remained elusive given that serial tumor biopsies are not routinely performed in adolescents and young adults. Sequencing of ctDNA samples will allow for exploration of temporal and spatial tumor heterogeneity. For example, these approaches have allowed for ctDNA-based identification of genomic copy-number changes specific to metastatic disease that were not present in primary tumor samples in patients with metastatic breast cancer [77]. Similarly, CTCs may prove to be an additional key analyte to explore tumor heterogeneity using a variety of single-cell genomic approaches.

While studies of osteosarcoma tumor biology using liquid biopsy are lacking, multiple studies of neuroblastoma have begun to demonstrate the promise of liquid biopsy for elucidating tumor genomic heterogeneity. Two studies utilizing a combination of whole-exome sequencing and targeted panel sequencing of plasma samples from patients with neuroblastoma demonstrated that (1) ctDNA provides an avenue to identify somatic mutations or copy-number changes associated with metastatic disease potential that may be missed when sequencing the primary tumor and (2) that sub-clonal events seen early in the disease course may become clonal events following treatment [78, 79]. Such an approach provides compelling evidence that deep sequencing of serial ctDNA samples from patients with osteosarcoma may deepen our understanding of spatial and temporal tumor heterogeneity and facilitate identification of driving events and markers of resistance to chemotherapy. As an increasing number of samples from patients with osteosarcoma are collected for ctDNA analysis, these questions are prime for exploration in the coming years.

The Path to Clinical Implementation

To date, liquid biopsy has not entered the clinical care of patients with osteosarcoma. Yet, as we have attempted to outline, liquid biopsy holds great promise for improving the care we provide to patients with osteosarcoma. We believe these assays may inform care across the clinical spectrum including early detection, diagnosis, risk stratification, on-therapy monitoring, detection of relapse, and detection of markers of resistance and sensitivity to therapy. A path to clinical implementation for liquid biopsy assays was outlined in a 2017 joint statement from the American Society of Clinical Oncology and College of American Pathologists [5]. Based on these recommendations, assays must demonstrate (1) analytic validity, or the ability to detect a targeted variant with accuracy, reproducibility, and reliability; (2) clinical validity, meaning the ability of the assay to divide a clinical group into multiple cohorts with significantly different outcomes; and finally (3) clinical utility, which means that knowledge gained from the assay can be used to significantly improve clinical care and outcomes. To date, only a handful of assays have gained regulatory approval in Europe and the United states for selection of patients for targeted therapies [7, 11, 12, 80–82]. No assays have gained regulatory approval for use in children. However, there are assays that are being utilized by clinicians for patient care, even without regulatory approval for a specific pediatric indications, most notably, the Guardant360 assay. At this time, the analytic validity, clinical validity, and clinical

utility of miRNA and CTC assays remain under investigation.

While there are a number of assays that have been developed for analysis of ctDNA, CTCs, and miRNAs in patients with osteosarcoma, for these to move toward the clinic, large prospective studies are needed that are sufficiently powered to demonstrate clinical validity. These studies could take the form of biology studies, in which patients receiving standard of care provide blood samples at prespecified time during their care. Furthermore, the inclusion of liquid biopsy studies on new clinical trials enrolling patients with osteosarcoma should be considered whenever possible. Given the rarity of osteosarcoma, these studies must necessarily be multicenter and require close collaboration. These studies can serve the basis for demonstrating clinical validity and then inform future therapeutic trials designed to improve outcomes.

Summary

In this chapter, we've attempted to outline the current state of liquid biopsy in oncology, pediatric oncology, and what has been done to bring liquid biopsy to patients with osteosarcoma. While ctDNA was described decades ago, and miRNAs and CTCs have been well established in other diseases, the study of liquid biopsy in osteosarcoma remains relatively new. Nevertheless, given early successes of liquid biopsy in diseases such as non-small cell lung cancer, as well as preliminary studies in osteosarcoma, we believe that these technologies may ultimately improve the care we provide to patients with osteosarcoma.

The evidence to date suggests that ctDNA, CTCs, and miRNAs are all ripe for analysis in patients with both localized and metastatic osteosarcoma at diagnosis and throughout treatment. The most mature clinical studies demonstrate that ctDNA may be a prognostic biomarker for patients with localized osteosarcoma. This is now being evaluated in a large multicenter study. Further studies of CTCs and miRNA in larger clinical studies will be key to determine how best these assays can inform clinical care.

References

1. Mandel P, Metais P (1948) Not Available. C R Seances Soc Biol Fil 142(3-4):241–243. http://www.ncbi.nlm.nih.gov/pubmed/18875018
2. Leon SA, Shapiro B, Sklaroff DM, Yaros MJ (1977) Free DNA in the serum of cancer patients and the effect of therapy. Cancer Res 37(3):646–650
3. Stroun M, Anker P, Lyautey J, Lederrey C, Maurice PA (1987) Isolation and characterization of DNA from the plasma of cancer patients. Eur J Cancer Clin Oncol 23(6):707–712. https://doi.org/10.1016/0277-5379(87)90266-5
4. Wan JCM, Massie C, Garcia-Corbacho J et al (2017) Liquid biopsies come of age: towards implementation of circulating tumour DNA. Nat Rev Cancer 17(4):223–238. https://doi.org/10.1038/nrc.2017.7
5. Merker JD, Oxnard GR, Compton C et al (2018) Circulating tumor DNA analysis in patients with cancer: American Society of Clinical Oncology and College of American Pathologists Joint Review. J Clin Oncol:JCO.2017.76.867. https://doi.org/10.1200/JCO.2017.76.8671
6. Abbou SD, Shulman DS, DuBois SG, Crompton BD (2019, (October 2018) Assessment of circulating tumor DNA in pediatric solid tumors: the promise of liquid biopsies. Pediatr Blood Cancer:e27595. https://doi.org/10.1002/pbc.27595
7. Sacher AG, Paweletz C, Dahlberg SE et al (2016) Prospective validation of rapid plasma genotyping for the detection of EGFR and KRAS mutations in advanced lung cancer. JAMA Oncol 2(8):1014–1022. https://doi.org/10.1001/jamaoncol.2016.0173
8. Warren JD, Xiong W, Bunker AM et al (2011) Septin 9 methylated DNA is a sensitive and specific blood test for colorectal cancer. BMC Med 9(1):133. https://doi.org/10.1186/1741-7015-9-133
9. Heitzer E, Haque IS, Roberts CES, Speicher MR (2019) Current and future perspectives of liquid biopsies in genomics-driven oncology. Nat Rev Genet 20(2):71–88. https://doi.org/10.1038/s41576-018-0071-5
10. Oxnard GR, Paweletz CP, Kuang Y et al (2014) Noninvasive detection of response and resistance in egfrmutant lung cancer using quantitative next-generation genotyping of cell-free plasma DNA. Clin Cancer Res 20(6):1698–1705. https://doi.org/10.1158/1078-0432.CCR-13-2482
11. Remon J, Caramella C, Jovelet C et al (2017) Osimertinib benefit in EGFR-mutant NSCLC patients with T790M-mutation detected by circulating tumour DNA. Ann Oncol 28(4):784–790. https://doi.org/10.1093/annonc/mdx017

12. Jenkins S, Yang JCH, Ramalingam SS et al (2017) Plasma ctDNA analysis for detection of the EGFR T790M mutation in patients with advanced non-small cell lung cancer. J Thorac Oncol 12(7):1061–1070. https://doi.org/10.1016/j.jtho.2017.04.003
13. Schmiegel W, Scott RJ, Dooley S et al (2017) Blood-based detection of RAS mutations to guide anti-EGFR therapy in colorectal cancer patients: concordance of results from circulating tumor DNA and tissue-based RAS testing. Mol Oncol 11(2):208–219. https://doi.org/10.1002/1878-0261.12023
14. Gröbner SN, Worst BC, Weischenfeldt J et al (2018) The landscape of genomic alterations across childhood cancers. Nature 555(7696):321–327. https://doi.org/10.1038/nature25480
15. Huether R, Dong L, Chen X et al (2014) The landscape of somatic mutations in epigenetic regulators across 1,000 paediatric cancer genomes. Nat Commun 5:1–7. https://doi.org/10.1038/ncomms4630
16. Lawrence MS, Stojanov P, Polak P et al (2013) Mutational heterogeneity in cancer and the search for new cancer-associated genes. Nature 499(7457):214–218. https://doi.org/10.1038/nature12213
17. Barris DM, Weiner SB, Dubin RA et al (2018) Detection of circulating tumor DNA in patients with osteosarcoma. Oncotarget 9(16):12695–12704. https://doi.org/10.18632/oncotarget.24268
18. Crompton BD, Stewart C, Taylor-Weiner A et al (2014) The genomic landscape of pediatric Ewing sarcoma. Cancer Discov 4(11):1326–1341. https://doi.org/10.1158/2159-8290.CD-13-1037
19. Tirode F, Surdez D, Ma X et al (2014) Genomic landscape of Ewing sarcoma defines an aggressive subtype with co-association of STAG2 and TP53 mutations. Cancer Discov 4(11):1342–1353. https://doi.org/10.1158/2159-8290.CD-14-0622
20. Perry JA, Kiezun A, Tonzi P et al (2014) Complementary genomic approaches highlight the PI3K/mTOR pathway as a common vulnerability in osteosarcoma. Proc Natl Acad Sci U S A 111(51):E5564–E5573. https://doi.org/10.1073/pnas.1419260111
21. Brohl AS, Solomon DA, Chang W et al (2014) The genomic landscape of the Ewing Sarcoma family of tumors reveals recurrent STAG2 mutation. PLoS Genet 10(7):e1004475. https://doi.org/10.1371/journal.pgen.1004475
22. Shern JF, Chen L, Chmielecki J et al (2014) Comprehensive genomic analysis of rhabdomyosarcoma reveals a landscape of alterations affecting a common genetic axis in fusion-positive and fusion-negative tumors. Cancer Discov 4(2):216–231. https://doi.org/10.1158/2159-8290.CD-13-0639
23. Kovac M, Blattmann C, Ribi S et al (2015) Exome sequencing of osteosarcoma reveals mutation signatures reminiscent of BRCA deficiency. Nat Commun. https://doi.org/10.1038/ncomms9940
24. Bousquet M, Noirot C, Accadbled F et al (2016) Whole-exome sequencing in osteosarcoma reveals important heterogeneity of genetic alterations. Ann Oncol 27(4):738–744. https://doi.org/10.1093/annonc/mdw009
25. Bayani J, Zielenska M, Pandita A et al (2003) Spectral karyotyping identifies recurrent complex rearrangements of chromosomes 8, 17, and 20 in osteosarcomas. Genes Chromosom Cancer 36(1):7–16. https://doi.org/10.1002/gcc.10132
26. Squire JA, Pei J, Marrano P et al (2003) High-resolution mapping of amplifications and deletions in pediatric osteosarcoma by use of CGH analysis of cDNA microarrays. Genes Chromosom Cancer 38(3):215–225. https://doi.org/10.1002/gcc.10273
27. Chen X, Bahrami A, Pappo A et al (2014) Recurrent somatic structural variations contribute to tumorigenesis in pediatric osteosarcoma. Cell Rep 7(1):104–112. https://doi.org/10.1016/j.celrep.2014.03.003
28. Pugh TJ, Morozova O, Attiyeh EF et al (2013) The genetic landscape of high-risk neuroblastoma. Nat Genet 45(3):279–284. https://doi.org/10.1038/ng.2529
29. Adalsteinsson VA, Ha G, Freeman SS et al (2017) Scalable whole-exome sequencing of cell-free DNA reveals high concordance with metastatic tumors. Nat Commun 8(1):1324. https://doi.org/10.1038/s41467-017-00965-y
30. Klega K, Imamovic-Tuco A, Ha G et al (2018) Detection of somatic structural variants enables quantification and characterization of circulating tumor DNA in children with solid tumors. JCO Precis Oncol 2018(2):1–13. https://doi.org/10.1200/PO.17.00285
31. Koelsche C, Hartmann W, Schrimpf D et al (2018) Array-based DNA-methylation profiling in sarcomas with small blue round cell histology provides valuable diagnostic information. Mod Pathol 31(8):1246–1256. https://doi.org/10.1038/s41379-018-0045-3
32. Shen SY, Singhania R, Fehringer G et al (2018) Sensitive tumour detection and classification using plasma cell-free DNA methylomes. Nature 563(7732):579–583. https://doi.org/10.1038/s41586-018-0703-0
33. Li W, Zhang X, Lu X et al (2017) 5-Hydroxymethylcytosine signatures in circulating cell-free DNA as diagnostic biomarkers for human cancers. Cell Res 27(10):1243–1257. https://doi.org/10.1038/cr.2017.121
34. Cayrefourcq L, Mazard T, Joosse S et al (2015) Establishment and characterization of a cell line from human circulating colon cancer cells. Cancer Res 75(5):892–901. https://doi.org/10.1158/0008-5472.CAN-14-2613
35. Zahn H, Steif A, Laks E et al (2017) Scalable whole-genome single-cell library preparation without pre-amplification. Nat Methods 14(2):167–173. https://doi.org/10.1038/nmeth.4140
36. Clark SJ, Lee HJ, Smallwood SA, Kelsey G, Reik W (2016) Single-cell epigenomics: powerful new methods for understanding gene regulation and cell identity. Genome Biol 17(1):72. https://doi.org/10.1186/s13059-016-0944-x

37. Kalinich M, Bhan I, Kwan TT et al (2017) An RNA-based signature enables high specificity detection of circulating tumor cells in hepatocellular carcinoma. Proc Natl Acad Sci 114(5):1123–1128. https://doi.org/10.1073/pnas.1617032114
38. Allard WJ, Matera J, Miller MC et al (2004) Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. Clin Cancer Res 10(20):6897–6904. https://doi.org/10.1158/1078-0432.CCR-04-0378
39. Vo KT, Edwards JV, Epling CL et al (2016) Impact of two measures of micrometastatic disease on clinical outcomes in patients with newly diagnosed Ewing Sarcoma: a report from the Children's Oncology Group. Clin Cancer Res. https://doi.org/10.1158/1078-0432.CCR-15-2516
40. Dubois SG, Epling CL, Teague J, Matthay KK, Sinclair E (2010) Flow cytometric detection of Ewing sarcoma cells in peripheral blood and bone marrow. Pediatr Blood Cancer 54(1):13–18. https://doi.org/10.1002/pbc.22245
41. Satelli A, Brownlee Z, Mitra A, Meng QH, Li S (2015) Circulating tumor cell enumeration with a combination of epithelial cell adhesion molecule- and cell-surface vimentin-based methods for monitoring breast cancer therapeutic response. Clin Chem 61(1):259–266. https://doi.org/10.1373/clinchem.2014.228122
42. Roth M, Linkowski M, Tarim J et al (2014) Ganglioside GD2 as a therapeutic target for antibody-mediated therapy in patients with osteosarcoma. Cancer 120(4):548–554. https://doi.org/10.1002/cncr.28461
43. Heiner JP, Miraldi F, Kallick S et al (1987) Localization of GD2-specific monoclonal antibody 3F8 in human osteosarcoma. Cancer Res 47(20):5401–5406
44. Hayashi M, Zhu P, McCarty G et al (2017) Size-based detection of sarcoma circulating tumor cells and cell clusters. Oncotarget 8(45):78965–78977. https://doi.org/10.18632/oncotarget.20697
45. Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116(2):281–297. https://doi.org/10.1016/s0092-8674(04)00045-5
46. Cortez MA, Bueso-Ramos C, Ferdin J, Lopez-Berestein G, Sood AK, Calin G (2011) a. MicroRNAs in body fluids--the mix of hormones and biomarkers. Nat Rev Clin Oncol 8(8):467–477. https://doi.org/10.1038/nrclinonc.2011.76
47. Montani F, Bianchi F (2016) Circulating cancer biomarkers: the macro-revolution of the micro-RNA. EBioMedicine 5:4–6. https://doi.org/10.1016/j.ebiom.2016.02.038
48. Chen X, Ba Y, Ma L et al (2008) Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res 18(10):997–1006. https://doi.org/10.1038/cr.2008.282
49. Sozzi G, Boeri M, Rossi M et al (2014) Clinical utility of a plasma-based miRNA signature classifier within computed tomography lung cancer screening: a correlative MILD trial study. J Clin Oncol 32(8):768–773. https://doi.org/10.1200/JCO.2013.50.4357
50. Montani F, Marzi MJ, Dezi F et al (2015) miR-test: a blood test for lung cancer early detection. J Natl Cancer Inst 107(6):djv063. https://doi.org/10.1093/jnci/djv063
51. Allen-Rhoades W, Kurenbekova L, Satterfield L et al (2015) Cross-species identification of a plasma microRNA signature for detection, therapeutic monitoring, and prognosis in osteosarcoma. Cancer Med 4(7):977–988. https://doi.org/10.1002/cam4.438
52. Shulman DS, Klega K, Imamovic-Tuco A et al (2018) 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 119(5):615–621. https://doi.org/10.1038/s41416-018-0212-9
53. Henderson TO, Whitton J, Stovall M et al (2007) Secondary sarcomas in childhood cancer survivors: a report from the Childhood Cancer Survivor Study. J Natl Cancer Inst 99(4):300–308. https://doi.org/10.1093/jnci/djk052
54. Amant F, Verheecke M, Wlodarska I et al (2015) Presymptomatic identification of cancers in pregnant women during noninvasive prenatal testing. JAMA Oncol 1(6):814–819. https://doi.org/10.1001/jamaoncol.2015.1883
55. Bianchi DW, Chudova D, Sehnert AJ et al (2015) Noninvasive prenatal testing and incidental detection of occult maternal malignancies. JAMA 314(2):162–169. https://doi.org/10.1001/jama.2015.7120
56. Dong J, Liu Y, Liao W, Liu R, Shi P, Wang L (2016) miRNA-223 is a potential diagnostic and prognostic marker for osteosarcoma. J Bone Oncol 5(2):74–79. https://doi.org/10.1016/j.jbo.2016.05.001
57. Ma W, Zhang X, Chai J, Chen P, Ren P, Gong M (2014) Circulating miR-148a is a significant diagnostic and prognostic biomarker for patients with osteosarcoma. Tumor Biol 35(12):12467–12472. https://doi.org/10.1007/s13277-014-2565-x
58. Bielack SS, Kempf-Bielack B, Delling G et al (2002) Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol 20(3):776–790. https://doi.org/10.1200/JCO.2002.20.3.776
59. Bacci G, Longhi A, Versari M, Mercuri M, Briccoli A, Picci P (2006) Prognostic factors for osteosarcoma of the extremity trerated with neoadjuvant chemotherapy: 15-year experience in 789 patients treated at a single institution. Cancer 106(5):1154–1161. https://doi.org/10.1002/cncr.21724
60. Parkinson CA, Gale D, Piskorz AM et al (2016) Exploratory analysis of TP53 mutations in circulating tumour DNA as biomarkers of treatment response for patients with relapsed high-grade serous ovarian carcinoma: a retrospective study. PLoS Med 13(12):e1002198. https://doi.org/10.1371/journal.pmed.1002198

61. Thierry AR, Mouliere F, Gongora C et al (2010) Origin and quantification of circulating DNA in mice with human colorectal cancer xenografts. Nucleic Acids Res 38(18):6159–6175. https://doi.org/10.1093/nar/gkq421
62. Bettegowda C, Sausen M, Leary RJ et al (2014) Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med 6(224):224ra24. https://doi.org/10.1126/scitranslmed.3007094
63. Lecomte T, Berger A, Zinzindohoué F et al (2002) Detection of free-circulating tumor-associated DNA in plasma of colorectal cancer patients and its association with prognosis. Int J Cancer 100(5):542–548. https://doi.org/10.1002/ijc.10526
64. Ziyan W, Shuhua Y, Xiufang W, Xiaoyun L (2011) MicroRNA-21 is involved in osteosarcoma cell invasion and migration. Med Oncol 28(4):1469–1474. https://doi.org/10.1007/s12032-010-9563-7
65. Duan Z, Choy E, Harmon D et al (2011) MicroRNA-199a-3p is downregulated in human osteosarcoma and regulates cell proliferation and migration. Mol Cancer Ther 10(8):1337–1345. https://doi.org/10.1158/1535-7163.MCT-11-0096
66. Osaki M, Takeshita F, Sugimoto Y et al (2011) MicroRNA-143 regulates human osteosarcoma metastasis by regulating matrix metalloprotease-13 expression. Mol Ther 19(6):1123–1130. https://doi.org/10.1038/mt.2011.53
67. Zhou G, Lu M, Chen J et al (2015) Identification of miR-199a-5p in serum as noninvasive biomarkers for detecting and monitoring osteosarcoma. Tumor Biol 36(11):8845–8852. https://doi.org/10.1007/s13277-015-3421-3
68. Lian F, Cui Y, Zhou C, Gao K, Wu L (2015) Identification of a plasma four-microRNA panel as potential noninvasive biomarker for osteosarcoma. PLoS One 10(3):e0121499. https://doi.org/10.1371/journal.pone.0121499
69. Tian Q, Jia J, Ling S, Liu Y, Yang S, Shao Z (2014) A causal role for circulating miR-34b in osteosarcoma. Eur J Surg Oncol 40(1):67–72. https://doi.org/10.1016/j.ejso.2013.08.024
70. Ouyang L, Liu P, Yang S, Ye S, Xu W, Liu X (2013) A three-plasma miRNA signature serves as novel biomarkers for osteosarcoma. Med Oncol 30(1):340. https://doi.org/10.1007/s12032-012-0340-7
71. Guenther LM, Rowe RG, Acharya PT et al (2017) Response Evaluation Criteria in Solid Tumors (RECIST) following neoadjuvant chemotherapy in osteosarcoma. Pediatr Blood Cancer:e26896. https://doi.org/10.1002/pbc.26896
72. Pearce MS, Salotti JA, Little MP et al (2012) Radiation exposure from CT scans in childhood and subsequent risk of leukaemia and brain tumours: a retrospective cohort study. Lancet 380(9840):499–505. https://doi.org/10.1016/S0140-6736(12)60815-0
73. McBride DJ, Orpana AK, Sotiriou C et al (2010) Use of cancer-specific genomic rearrangements to quantify disease burden in plasma from patients with solid tumors. Genes Chromosomes Cancer 49(11):1062–1069. https://doi.org/10.1002/gcc.20815
74. McDonald BR, Contente-Cuomo T, Sammut S-J et al (2019) Personalized circulating tumor DNA analysis to detect residual disease after neoadjuvant therapy in breast cancer. Sci Transl Med 11(504):eaax7392. https://doi.org/10.1126/scitranslmed.aax7392
75. Ozaki T, Schaefer KL, Wai D et al (2002) Genetic imbalances revealed by comparative genomic hybridization in osteosarcomas. Int J Cancer 102(4):355–365. https://doi.org/10.1002/ijc.10709
76. Roberts RD, Lizardo MM, Reed DR et al (2019) Provocative questions in osteosarcoma basic and translational biology: a report from the Children's Oncology Group. Cancer 36(16):1631–1641. https://doi.org/10.1002/cncr.32351
77. Stover DG, Parsons HA, Ha G et al (2018) Association of cell-free DNA tumor fraction and somatic copy number alterations with survival in metastatic triple-negative breast cancer. J Clin Oncol 36(6):543–553. https://doi.org/10.1200/JCO.2017.76.0033
78. Chicard M, Boyault S, Daage LC et al (2016) Genomic copy number profiling using circulating free tumor DNA highlights heterogeneity in Neuroblastoma. Clin Cancer Res 22(22):5564–5573. https://doi.org/10.1158/1078-0432.CCR-16-0500
79. Chicard M, Colmet Daage L, Clement N et al (2017) Whole exome sequencing of cell-free DNA reveals temporo-spatial heterogeneity and identifies treatment-resistant clones in neuroblastoma. Clin Cancer Res. clincanres.1586.2017. https://doi.org/10.1158/1078-0432.CCR-17-1586
80. Cobas EGFR Mutation test V2 - PMA Number: P150044. https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfpma/pma.cfm?id=P150044. Published 2016
81. EMA. European Medicines Agency. Iressa: public assessment report—product information. https://www.ema.europa.eu/en/documents/product-information/iressa-epar-product-information_en.pdf. Published 2016
82. EMA. European Medicines Agency. Tagrisso: public assessment report—product information