

Establishment of tumor-specific copy number alterations from plasma DNA of patients with cancer

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With the increasing number of available predictive biomarkers, clinical management of cancer is becoming increasingly reliant on the accurate serial monitoring of tumor genotypes. We tested whether tumor-specific copy number changes can be inferred from the peripheral blood of patients with cancer. To this end, we determined the plasma DNA size distribution and the fraction of mutated plasma DNA fragments with deep sequencing and an ultrasensitive mutation-detection method, *i.e.*, the Beads, Emulsion, Amplification, and Magnetics (BEAMing) assay. When analyzing the plasma DNA of 32 patients with Stage IV colorectal carcinoma, we found that a subset of the patients (34.4%) had a biphasic size distribution of plasma DNA fragments that was associated with increased circulating tumor cell numbers and elevated concentration of mutated plasma DNA fragments. In these cases, we were able to establish genome-wide tumor-specific copy number alterations directly from plasma DNA. Thus, we could analyze the current copy number status of the tumor genome, which was in some cases many years after diagnosis of the primary tumor. An unexpected finding was that not all patients with progressive metastatic disease appear to release tumor DNA into the circulation in measurable quantities. When we analyzed plasma DNA from 35 patients with metastatic breast cancer, we made similar observations suggesting that our approach may be applicable to a variety of tumor entities. This is the first description of such a biphasic distribution in a surprisingly high proportion of cancer patients which may have important implications for tumor diagnosis and monitoring.

Recent advances in the understanding of the molecular mechanisms of cancer have highlighted the need for personalized medicine approaches not only in terms of prognosis but also for diagnostic strategies. Extensive work has resulted in the identification of biomarkers that have been implemented in cancer clinical practice at several levels: prognostics, predictive and pharmacokinetic biomarkers. For

example, in colorectal cancer (CRC), the *KRAS* mutations in Exon 2 (Codons 12 and 13) represent a paradigm that has been established as a negative predictive marker for treatment with epidermal growth factor receptor (EGFR) inhibitors such as cetuximab and panitumumab.¹ The discovery of further biomarkers will be accelerated by the recent advances in cancer genomics.

Key words: plasma DNA, tumor monitoring, predictive and prognostic biomarker, copy number changes

Additional Supporting Information may be found in the online version of this article.

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What's new?

Tumors shed their DNA into the bloodstream. This DNA can be detected, but whether it's useful as a diagnostic tool hasn't been clear from existing reports. Rather than attempt to pick out specific mutations, however, this study asked whether it would be possible to get a genome-wide view of tumor-specific copy number changes from this circulating tumor cell DNA. When they analyzed the plasma DNA of patients with colorectal cancer, the authors found that about a third of the patients had plasma DNA that fell into two distinct size categories, and this correlated with higher numbers of circulating tumor cells. They could then detect tumor-specific copy-number changes from this plasma DNA. Further developing this non-invasive acquisition of tumor material could aid in tailoring specific disease treatment strategies.

Access to accurate and sensitive methods for the detection of such predictive biomarkers is of utmost importance to the clinical oncologist. Numerous studies have tried to identify such biomarkers in the form of circulating DNA because tumors shed DNA into the circulation that can be detected by appropriate means. However, early reports proposing that the presence or absence of circulating DNA or its concentration was of diagnostic value^{2,3} have been called into question.⁴⁻⁷ Analyses of plasma/serum DNA for loss of heterozygosity^{8,9} or for tumor-related methylation patterns^{10,11} often lack specificity. Furthermore, it has been proposed that the detection of mutations in peripheral blood, which have previously been identified in the corresponding primary tumor from the same patient, may provide a specific biomarker of disease burden. Therefore, multiple studies focused on the detection of such specific and predetermined mutations.¹²⁻¹⁸ For example, the emergence of *KRAS* mutant clones as evidence for acquired resistance to targeted EGFR blockade in patients with CRC has been inferred from the analysis of plasma DNA.^{19,20} A broader approach is the use of targeted amplicon sequencing for the simultaneous analysis of multiple cancer driver genes.²¹ However, the sensitive and specific detection of a mutated base in a vast excess of normal DNA requires specialized techniques that are currently beyond the scope of many diagnostic laboratories.^{12-14,18}

In our study, we investigated whether instead of a targeted approach a genome-wide view of tumor-specific copy number changes can be established from peripheral blood, *i.e.*, plasma DNA from patients with cancer. To this end, we simultaneously quantified the normal and mutant DNA molecules in a given sample and established genome-wide copy number changes from plasma and compiled detailed data on copy number changes in relation to the respective primary tumors and metastases. We validated these observations with blood samples from 35 patients with breast cancer. Our results suggest that complex tumor genomes can be reconstructed from the peripheral blood of patients with cancer. Our approach may be of interest for two scenarios: first, as a research tool to investigate a tumor genome at late stage disease, which is not commonly studied. Second, it may pave the way for new disease monitoring strategies.

Material and Methods**Preparation of plasma DNA**

Whole blood (9 ml) was collected in routine ethylenediamine tetraacetic acid (EDTA) Vacutainer tubes (BD Biosciences, Heidelberg, Germany). To stabilize cell membranes and to

impede cell lysis, 0.225 ml of a 10% neutral buffered solution containing formaldehyde (4% weight per volume) (Sigma-Aldrich, Vienna, Austria) was added immediately after blood withdrawal. Blood samples were gently inverted, stored at room temperature and further processed within 2 hr. Plasma was prepared according to Ref. 22. In brief, tubes were centrifuged at 200g for 10 min with the brake and acceleration powers set to zero, followed by a subsequent centrifugation step at 1600g for 10 min. The supernatant was collected, transferred to a new 15 ml tube and spun at 1600g for 10 min. The plasma was carefully aliquoted into new 2 ml Eppendorf tubes and stored at -80°C .

DNA was isolated from plasma samples using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) or the Qiagen Circulating Nucleic Acids Kit (CNA) (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was eluted in 30 μl of distilled water for Mini Kit extractions and in 100 μl for CNA Kit, respectively.

Further methods

All other methods used in this article are detailed in the Supporting Information.

Results**A subset of patients with CRC had a biphasic plasma DNA size distribution**

We analyzed the peripheral blood of 32 patients with advanced stage CRC (Supporting Information and Supporting Information Table 1). We started with measuring of the plasma DNA concentrations of healthy controls and patients with advanced-stage CRC. Compared to controls (mean: 15.21 ng/ml; median: 14.37 ng/ml; range: 12.20–19.51 ng/ml), patients showed invariably higher values with substantial variability (mean: 275.35 ng/ml; median: 139.0 ng/ml; range: 22.44–1,037.49 ng/ml) ($p < 0.0001$).

Because mutant DNA fragments in the blood circulation of cancer patients were reported to be degraded relative to non-mutant DNA fragments,¹² we used a microfluidics-based platform for sizing. We observed an enrichment of plasma DNA fragments within the range of 85–230 bp in the healthy controls (Fig. 1a, upper plot). Plasma DNA fragments within this size range have been associated with the release of DNA from apoptotic cells after enzymatic processing.^{12,23} There was no significant qualitative difference regarding the size distribution of plasma DNA fragments between the healthy controls and

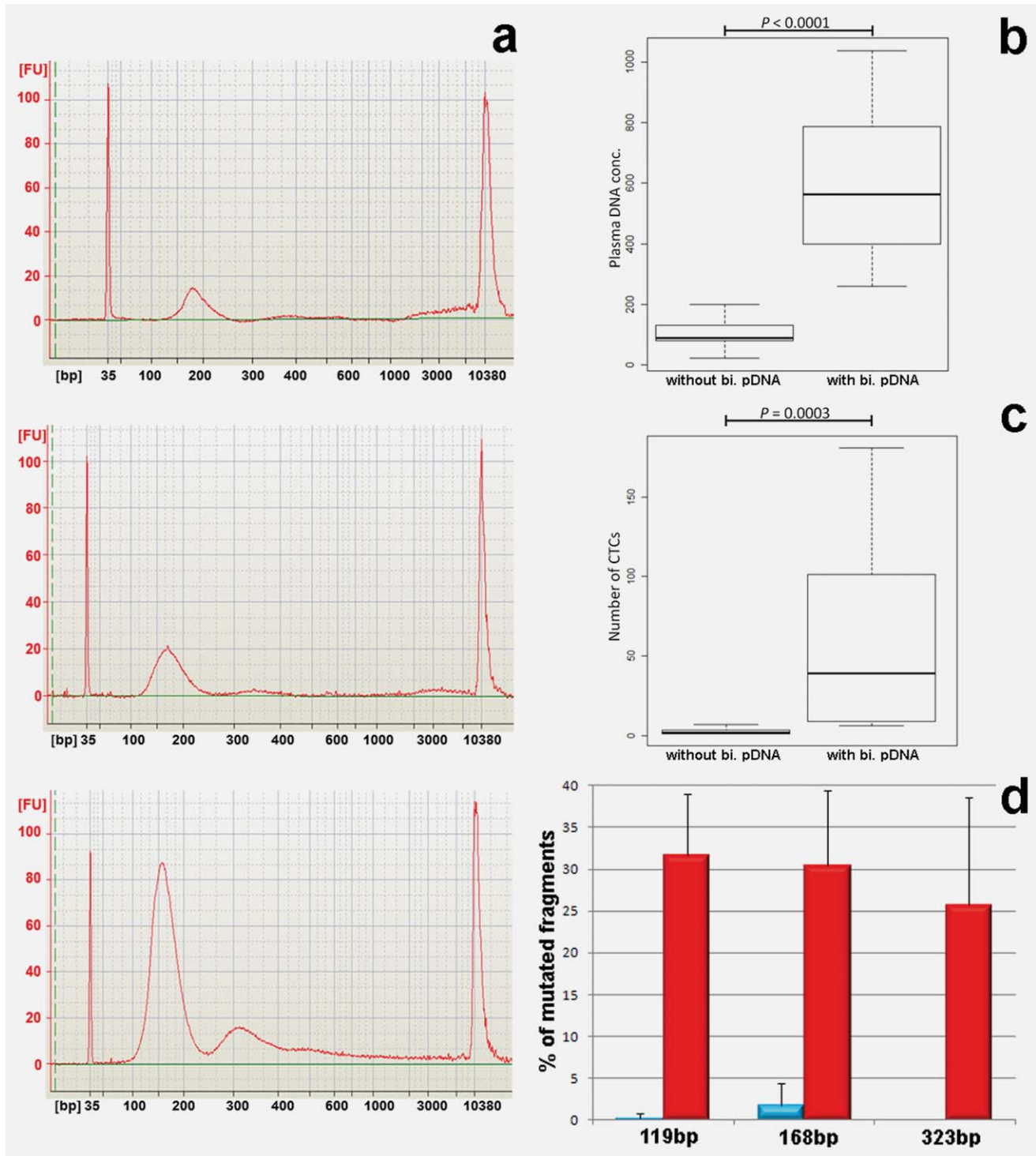


Figure 1. Characteristics of plasma DNA from healthy controls and patients with advanced-stage colorectal cancer (CRC). (a) Size distribution of plasma DNA fragments from a healthy donor (upper plot), Patient #11 (center plot) and Patient #22 (lower plot). Normalization was performed using two internal markers, visible as high, narrow amplitudes at Positions 35 and 10,380 bp, respectively. For each analysis, 800 pg of DNA was used. (b) Patients with a biphasic plasma DNA size distribution (with bi. pDNA) have higher plasma DNA concentrations compared to patients lacking the second peak (without bi. pDNA). (c) The occurrence and number of CTCs is closely correlated with a biphasic plasma DNA size distribution. (d) Deep sequencing using three different sequencing reaction sizes (*i.e.*, 119, 168 and 323 bp) identified few mutated *KRAS* fragments in patients without a biphasic plasma DNA size distribution (blue), but high levels of mutated *KRAS* fragments in patients with a biphasic plasma DNA size distribution (red) (the errors bars represent SDs).

21 (65.6%) of the CRC cases despite the higher concentrations of plasma DNA in the latter group (Fig. 1a, center plot). However, in 11 (34.4%) patients, we observed a second peak consisting of DNA fragments with a size range of 240 to 400 bp, or even longer in some cases (Fig. 1a, lower plot). Interestingly, the patients with biphasic plasma DNA size distributions had significantly higher plasma DNA concentrations (mean: 604 ng/ml; median: 562 ng/ml; range: 260–1,037 ng/ml) than patients without a second peak (mean: 103 ng/ml; median: 89 ng/ml; range: 22–201 ng/ml) ($p < 0.0001$; Fig. 1b).

The biphasic plasma DNA size distribution correlates with circulating tumor cell occurrence

To investigate whether the DNA size distributions detected might reflect the contributions of different tumor cell populations releasing their DNA into the circulation around the time of blood collection, we determined the number of circulating tumor cells (CTCs). We used the FDA-approved Veridex system for CTC detection²⁴ in 30 of the 32 patients (the analysis could not be performed for patients #10 and #35). We observed a clear correlation between the presence of a biphasic plasma DNA size distribution and CTC occurrence. In the patient group with a biphasic distribution ($n = 10$), we found a mean number of 52 CTCs (median: 35; range: 0–181). In contrast, the mean CTC number in patients without a biphasic plasma DNA size distribution ($n = 20$) was only 1.5 (median: 1; range: 0–7) ($p = 0.0003$; Fig. 1c).

Deep sequencing of plasma DNA for KRAS mutations

To investigate whether mutant DNA fragments were present in both the first and second peaks in the plasma DNA fragment size distribution, we used ultra-deep pyrosequencing. We used eight patient samples with and without biphasic plasma DNA size distributions and KRAS mutations in their corresponding primary tumors to establish the percentage of mutated plasma DNA fragments for different sequencing reaction sizes (*i.e.*, 119, 168 and 323 bp). In four patients (#6, #10, #25 and #38) with biphasic plasma DNA size distributions, we found a high percentage of mutated DNA fragments compared to the other four patients without biphasic peak (#7, #11, #15 and #16) (Fig. 1d; Supporting Information Table 2). With the exception of Patient #25, the fraction of mutant molecules was not dependent on the size of the amplicon, suggesting that for these patients (#6, #10 and #38) DNA from the first and the second peaks consists largely of tumor DNA. By contrast, in the four patients who had only the first apoptosis-related peak deep sequencing identified mutated KRAS fragments at low levels in two cases (#11 and #15) and none in the other two patients (#7 and #16) (Fig. 1d; Supporting Information Table 2). In these four patients, mutated DNA fragments were not observed in the 323 bp sequencing reaction.

Genome-wide estimation of copy number changes in plasma DNA

We postulated that, in addition to KRAS-mutated DNA fragments, other tumor DNA fragments should be present in the

circulation of these patients and that these might provide insights about the tumor genome. To address this hypothesis, we generated random DNA libraries by converting the plasma DNA fragments into polymerase chain reaction (PCR)-amplifiable OmniPlex Library molecules flanked by universal priming sites for whole-genome amplification (WGA) and subjected the WGA products to array CGH on a 60 K microarray platform. This array platform consists of 55,077 oligonucleotides, and we calculated for each of these oligonucleotides whether the ratio values were decreased, balanced or increased. Plasma DNA from six healthy controls showed a mean of 4,026 oligonucleotides from the 55,077 oligonucleotides (7.2%; range: 3,606–4,321; 6.4%–7.7%), with aberrant ratio values for the autosomes (Fig. 2a) (details about these calculations are in the Supporting Information). When we performed array CGH with plasma DNA from patients who had only the first plasma DNA peak ($n = 21$), we observed an increase in oligonucleotides with aberrant ratio values with a mean of 5,572 oligonucleotides (10.0%; range: 4,587–7,469; 8.2%–13.4%; $p = 0.001$ compared to the healthy controls) (Fig. 2b). Because these oligonucleotides involved only single, nonadjacent oligonucleotides, they most likely represented artifacts of the fragmentation and/or amplification process. In contrast, the plasma DNA in 10 of the 11 patients with a second peak had a mean number of oligonucleotides with copy number changes of 12,476 (22.3%; range: 9,117–14,915; 16.3%–26.7%), which differed highly significantly from both the aforementioned CRC cases and the healthy controls ($p < 0.0001$ each) (Fig. 2c).

Tumor-specific, genome-wide imbalances in plasma DNA with a biphasic size distribution

Because most of the aforementioned copy number changes in the plasma DNA with biphasic size distribution involved large regions of adjacent oligonucleotides, we determined whether or not they were tumor specific by comparing them to copy number changes of the primary tumor and, if available, metastatic material. As an exemplary case, we present Patient #6 in greater detail. When the initial diagnosis was made in Patient #6, he already had metastases in the liver, bones, abdominal lymph nodes and peritoneum. At this time, only a biopsy was obtained from the primary tumor. Five months later, a cerebellar metastasis was completely resected, and 3 months after that, we collected blood for our analyses—*i.e.*, 8 months after the initial diagnosis. Thus, multiple tumor sites could have released tumor DNA into the circulation at the time of blood collection.

We noted marked copy number differences between the primary tumor and the cerebellar metastasis in Patient #6 (Supporting Information Fig. 1, Panels 1 and 2), indicative of the presence of several malignant cell clones. We found numerous copy number changes in the plasma DNA (Supporting Information Fig. 1, Panel 3) and performed a detailed analysis consisting of the following steps: First, we compared the ratio of the profiles of the available material, *i.e.*, primary colorectal tumor, cerebellar metastasis and plasma DNA (Fig.

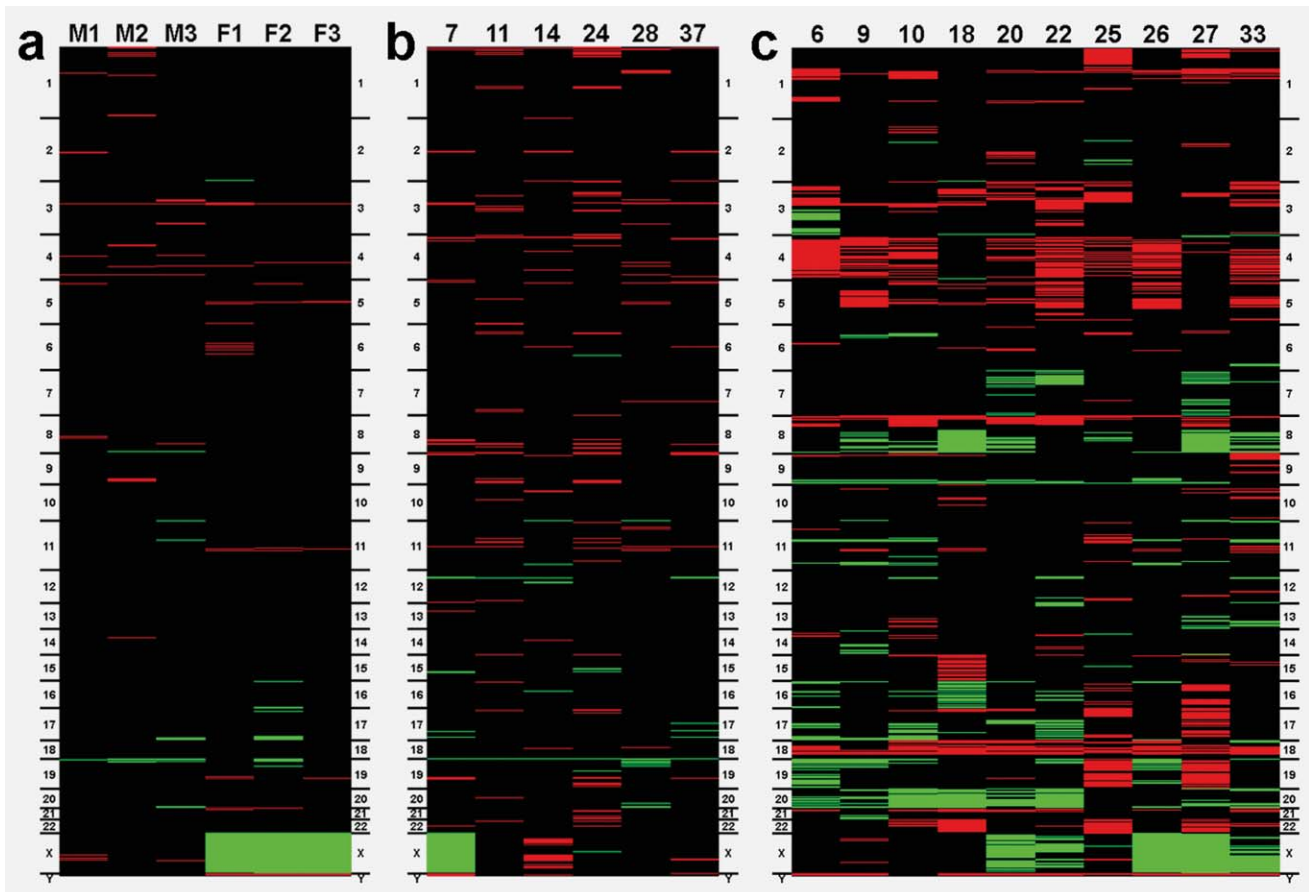


Figure 2. Heat maps of plasma DNA profiles from healthy controls and patients with advanced-stage colorectal cancer (CRC). (a) Heat map of plasma DNA profiles from healthy donors (black: balanced; red: under-represented; green: over-represented). As we used male reference DNA in all experiments, female plasma DNA samples have a relative over-representation of the X chromosome and an under-representation of the Y-chromosome (F1–F3), whereas male samples have balanced sex chromosomes (M1–M3). (b) Heat maps of six exemplary patients (#7, #11, #14, #24, #28 and #37) that lack a biphasic plasma DNA size distribution. (c) Heat maps of plasma DNA from 10 patients (#6, #9, #10, #18, #20, #22, #25, #26, #27 and #33) that have a biphasic plasma DNA size distribution.

3a). We then constructed detailed heat maps showing whether the copy number status was decreased, balanced or increased for each oligonucleotide on our array platform (Fig. 3b). Finally, we determined whether the copy number status for each oligonucleotide occurred only in the plasma DNA or also in the primary tumor and/or metastasis (Fig. 3c). These analyses revealed that the copy number status of 46.3% of the oligonucleotides on our array platform was present in all three lesions; 18.1% was shared by the primary tumor and plasma DNA, 18.0% was shared by the metastasis and plasma DNA and 17.6% was unique to the plasma DNA (Fig. 3c). This suggested that the observed changes in the plasma DNA were tumor-specific and were largely caused by clones both from the primary and the cerebellar metastatic tumor. As the cerebellar metastasis had been removed, cells from this metastatic clone are apparently still present in the patient. Whether the copy number changes observed only in the plasma DNA could have resulted from the other metastases or from parts of the primary tumor that were not included in our analysis remains unknown.

In addition to Patient #6, we had three other patients (*i.e.*, #9: Supporting Information Fig. 2; #26: Supporting Information Fig. 3; #33: Supporting Information Fig. 4) for whom material from the primary tumor and metastases were available for comparison. Altogether, the plasma DNA samples from these four patients (*i.e.*, #6, #9, #26 and #33) displayed an identical copy number status (*i.e.*, lost, balanced and gained) in all three samples (primary, metastasis and plasma DNA) for an average of 54.7% (median: 54.3%; range: 46.3%–63.6%) of all oligonucleotides. About 10% (median: 9.4%; range: 3.1%–18.1%) of plasma DNA copy number changes were unique to the metastasis and, *vice versa*, ~14.5% (median: 16.2%; range: 2.9%–22.7%) were only present in the primary tumor. About 20.9% (median: 18.7%; range: 15.7%–30.4%) of plasma DNA changes were observed only in the plasma DNA but not in the primary tumor and metastasis. However, all four patients had additional metastases at various sites (Supporting Information Table 1), which were not accessible to us. Thus, copy number changes observed only in the plasma DNA could reflect alterations from a metastatic site not included in our analysis.

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