

Tumor Cell-Free DNA Copy Number Instability Predicts Therapeutic Response to Immunotherapy



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Abstract

Purpose: Chromosomal instability is a fundamental property of cancer, which can be quantified by next-generation sequencing (NGS) from plasma/serum-derived cell-free DNA (cfDNA). We hypothesized that cfDNA could be used as a real-time surrogate for imaging analysis of disease status as a function of response to immunotherapy and as a more reliable tool than tumor biomarkers.

Experimental Design: Plasma cfDNA sequences from 56 patients with diverse advanced cancers were prospectively collected and analyzed in a single-blind study for copy number variations, expressed as a quantitative chromosomal number instability (CNI) score versus 126 noncancer controls in a training set of 23 and a blinded validation set of 33. Tumor biomarker concentrations and a surrogate marker for T regulatory cells (Tregs) were comparatively analyzed.

Results: Elevated CNI scores were observed in 51 of 56 patients prior to therapy. The blinded validation cohort provided an overall prediction accuracy of 83% (25/30) and a positive predictive value of CNI score for progression of 92% (11/12). The combination of CNI score before cycle (Cy) 2 and 3 yielded a correct prediction for progression in all 13 patients. The CNI score also correctly identified cases of pseudo-tumor progression from hyperprogression. Before Cy2 and Cy3, there was no significant correlation for protein tumor markers, total cfDNA, or surrogate Tregs.

Conclusions: Chromosomal instability quantification in plasma cfDNA can serve as an early indicator of response to immunotherapy. The method has the potential to reduce health care costs and disease burden for cancer patients following further validation. *Clin Cancer Res*; 1–8. ©2017 AACR.

Introduction

The assessment of efficacy of systemic antitumor therapy is essential for the optimal management of cancer patients. Current assessments are accomplished with imaging procedures at defined intervals evaluated using standard (RECIST 1.1) criteria (1) with or without frequent measurement of protein humoral tumor markers. Depending on the localization of the tumor, other procedures, such as ultrasound, can be useful. Nevertheless, there are numerous limitations. Tumor markers may be absent or variably expressed. Imaging may be influenced by nontumor burden factors such as inflammatory responses in the microenvironment of the neoplasm. Early assessment of response to immunotherapy remains an unmet need to discern response/pseudo-tumor progression (2, 3) from progression/hyperprogression (4).

Tumor genomic testing to guide treatment decisions continues to evolve since its introduction into clinical practice matching specific cancers with targeted therapies. Genomic testing of representative tumor biopsies provides mutational status of known driver genes, but methods currently available are reported to be inconsistent (5–7). Sequential biopsies during therapy have been proposed to capture alterations in driver mutations occurring with selection pressures from systemic therapies (8), which is often contraindicated due to technical or safety issues and patient reluctance. As an alternative, "liquid biopsies," utilizing tumor-derived cell-free DNA (cfDNA) present in plasma can be used (9) as a minimally invasive method to identify "actionable" mutations with their inherent limitations, for example, frequencies of such mutations being only 50% or lower (10), and conflicting results by technology (6, 7).

One hallmark of cancer biology is that most cancer cells have chromosomal instability often expressed as large physical or functional somatic gains and losses in tumor cfDNA (11–13). This report describes a novel application of the liquid biopsy by quantifying cancer-related chromosomal instability in cfDNA, an approach minimally influenced by clonal heterogeneity, which remains a problem for targeted "actionable" genes (10). The quantification of chromosomal instability from a liquid biopsy notably does not require any prior knowledge of individual somatic tumor mutations simplifying its use in clinical settings.

In this single-blind study of patients with multiple solid tumor types during immunotherapy treatment, alone or in combination

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Translational Relevance

Genomic analysis of tissue malignancies is well recapitulated by tumor cell-free DNA (cfDNA) using the power of next-generation sequencing (NGS). This study demonstrates that more than 90% of nonresectable diverse human cancers had NGS detectable "hot spots" of chromosomal instabilities in cfDNA from a "liquid biopsy." Using a classical training and a blinded prospective validation subset, the data provide a statistically high concordance of quantified chromosomal instability (CNI score) and the prediction of stable versus progressive disease as a function of immune-based therapy prior to standard imaging analysis. The data further demonstrate the clear superiority of cfDNA analysis versus classical protein tumor markers used for progression prediction. This is the first report of the real-time advantage of tumor cfDNA analysis of chromosomal instability in the evaluation of drug efficacy versus RECIST imaging criteria.

with systemic chemotherapy (ImmunoTx), the changes in genomic copy number instability (CNI) score of cfDNA (13) during the first therapeutic cycles was compared with conventional humoral tumor markers, with the aim to assess the efficacy of therapy as defined by imaging based RECIST1.1 and irRECIST (14). The primary objectives of this study were to (i) investigate the usefulness of cfDNA CNI scores in different tumor types for early prediction of therapy efficacy and (ii) determine equivalency to conventional biochemical tumor markers. Secondary objectives were the evaluation of total cfDNA concentrations and percentage of peripheral Tregs for the same purpose.

Materials and Methods

Study design

The study was conducted as a prospective training/validation study. After obtaining written informed consent, 56 patients with advanced cancer were included at Western Regional Medical Center (WRMC, Goodyear, AZ), with blood collection and usage for this study approved by the Western Institutional Review Board (protocol no. 20140257). The study was conducted in accordance with the Declaration of Helsinki. All patients underwent systemic treatment for their advanced cancer including imaging reviewed by board-certified radiologists. Patients were divided into a Discovery cohort consisting of the first 23 patients of which 11 were assessed as disease progression (PD) and 12 as disease controlled (DC), which includes stable disease (SD) and partial response (PR). The validation cohort consisted of a subsequent prospective collection of 33 patients (Supplementary Tables S1 and S2). All patients received either standard commercially available immunotherapy (IL2, PD-1 inhibitor, and/or CTLA-4 inhibitor), or were receiving a combination of a PD-1 inhibitor with systemic chemotherapy, targeted therapy, and/or radiotherapy as part of a treatment clinical trial. Blood for cfDNA was collected before the first cycle of treatment and before each consecutive cycle. Tumor markers (TuM) were quantified as standard of care, where applicable, by the WRMC CLIA-certified clinical laboratory.

Computation of irRECIST and RECIST 1.1 ($n = 56$) was performed by a board-certified medical oncologist (G.J. Weiss). Patients that did not have a response scan due to clinical dete-

rioration or had clinical deterioration between subsequent response scans, were categorized as PD, and where applicable, further defined as clinical PD and/or biochemical PD (using conventional protein tumor markers). One ovarian cancer patient (ID no. 13-38-154) showed a steeply rising CA-125, and subsequent clinical deterioration, but no radiologic change at the time. Subsequent imaging indicated misclassification on prior imaging and was categorized as PD.

Blood samples were anonymized and coded by a unique patient identifier and time point of draw. Thus, CNI scores were determined without prior knowledge of the clinical status. At the end of the training and validation phases, imaging data were assessed for study purposes and the CNI results were unblinded together with the treatment response estimates and compared. The values of tumor markers were kept undisclosed throughout the study and were added to the database after evaluation of the Validation group to ensure a bias-free comparison with the CNI score results.

Processing of plasma and extraction of cfDNA

Blood was collected in Cell-Free DNA BCT tubes (Streck), which inhibits plasma DNase activity and preserves leukocyte integrity (15), and processed within 5 days. Tubes were centrifuged at $2,500 \times g$ for 10 minutes to separate the plasma from the peripheral blood cells. Plasma was stored at -80°C and shipped to the central laboratory of Chronix (Göttingen, Germany). DNA was extracted from 2-mL aliquots of plasma using the Large Volume Viral Nucleic Acids Extraction Kit (Roche) according to the manufacturer's instructions but without the addition of carrier RNA. An artificial spike-in DNA was added to each sample before extraction to assess extraction efficiency. The DNA was eluted into 50 μL low-TE buffer (EDTA+Na 0.05 mmol/L, Tris 5 mmol/L) in DNA low bind tubes (Sarstedt) and stored at -20°C until sequencing library preparation.

DNA recovery quantification from plasma

For the quantification of absolute cfDNA two assays, each targeting one single copy genomic locus was used in one droplet digital (ddPCR) together with one assay targeting the artificial spike product. The ddPCR was carried out in $1 \times$ ddPCR Supermix for Probes (Bio-Rad) and contained the following primers and probes: (i) genomic assays: S55.F, S55.R, S110.F, and S110.R (0.6 $\mu\text{mol/L}$ each), S55.ProbeA, S55.ProbeB, S110.ProbeA, and S110.ProbeB (0.15 $\mu\text{mol/L}$ each); (ii) spike assay: Spike.F and Spike.R (0.6 $\mu\text{mol/L}$ each) and Spike.Probe (0.3 $\mu\text{mol/L}$; Supplementary Table S3). Cycling conditions were initial denaturation 95°C for 10 minutes; 40 cycles 95°C for 30 seconds, 55°C for 1 minute; final heat stabilization 98°C for 10 minutes. The absolute cfDNA concentrations were corrected for the determined spike recovery rate and a PCR length-based efficiency of 60% for the 90 bp amplicons.

Sequencing

Samples with cfDNA yields >10 ng were processed using the ThruPLEX DNA-seq Kit (Rubicon Genomics) according to the manufacturer's instructions. The resulting sequencing libraries were pooled and paired-end sequenced (37bp/36bp) on a Next-Seq500 (Illumina).

CNI score calculation

Copy numbers were called after mapping using BWA (16) and quality filtering in approximately 5.5 Mbp windows (sliding),

yielding an average read coverage of 24,000-fold per bin. Reads with a mapping quality score not reaching the maximum value in BWA were censored from further analysis. After correction for GC content and mappability using proprietary algorithms for cfDNA sequencing, the read counts were transformed into log₂ ratios (17) and converted into Z-values based on Gaussian transformations versus a normal control group ($n = 126$). These secondary data were then subjected to a noise-reducing proprietary bioinformatics pipeline using stochastic and statistic algorithms to calculate a final Z-score for each bin value to be within the dispersion of the normal control group (null hypothesis: equality). For bins of which the null hypothesis is rejected at a 0.2% false positive rate, the corresponding absolute values of the Gaussian cumulative density function are summed to generate the CNI score as general measure of tumor-derived copy number instability.

CNI technical reproducibility

Technical controls consisted of (i) a plasma pool of healthy individuals, (ii) sheared tumor DNA with known copy number imbalances mixed at 1% and 10% in normal PBMC DNA from the same patient, and (iii) sheared to 200 bp (average). All three control pools were prepared and analyzed analogous to the study samples. CNI values (between runs) of the normal plasma pool (5th–95th percentile range: 4–31) were within the range of the normal control group mentioned above ($n = 126$ 5th–95th percentile range: 0–25). CNI values (25 runs) were (average \pm SD) 70 ± 13 for 1% and 219 ± 16 for 10% tumor DNA, both above the 97.5th percentile of 126 healthy controls (CNI score = 30). Controls were included in each run and had to be within the ± 2 SD range for run quality acceptance.

Treg-specific demethylation region percentage

The Treg-specific demethylation region (TSDR) of FoxP3 was measured after bisulfite conversion of 500 ng PBMC DNA using the EZ-DNA Methylation-Gold Kit (Zymo Research). Of the 12- μ L eluate, 1 μ L was used for determination of methylated region, whereas 4 μ L were used for determination of demethylated region. Each reaction contained 1 \times ddPCR Supermix for probes (Bio-Rad), 0.75 μ mol/L of each primer, and 0.25 μ mol/L of the respective probe (Supplementary Table S3) in a total reaction

volume of 20 μ L. Droplets were read in the QX100 or QX200 Droplet Digital PCR System (Bio-Rad) and analyzed using the embedded QuantaSoft software. Results were expressed as percent copies TSDR of total FoxP3 in a subset of 29 patients.

Statistical and analytic plans

Microsoft Excel was used for statistical calculations and data plots. Significance of contingencies were calculated using Fisher exact test and frequencies are reported by proportions and the exact Clopper–Pearson method was used for confidence intervals (CI) given as 5th to 95th percentile (16).

All samples were prospectively collected and cfDNA analytics were done continuously and immediately recorded into a database. When the clinical outcome (irRECIST and RECIST 1.1) of the first 23 enrolled patients was available, the outcome data of those first 23 patients were unblinded and used for developing of the prediction method, while continuing to enroll patients under the conditions above, until a final total of 56 patients were recruited. The method set using the discovery group was applied to the remaining 33 patients for cfDNA-based outcome prediction and the clinical outcome was unblinded for comparative statistical analyses.

Results

All 56 study patients with metastatic cancer had plasma samples collected prior to initiation of ImmunoTx for up to six cycles of treatment. Five patients were censored from further analysis due to low CNI scores at baseline (two melanoma in the Discovery cohort, two renal and one pancreatic cancer in the Validation cohort). Elevated CNI scores prior to therapy were seen in the 51 evaluable patients (91% CI: 82%–97%), when compared to the 95th percentile of noncancerous controls (Fig. 1), which is a substantially higher rate compared with SNP-based approaches (10). Patients with carcinoma of the lung yielded the highest average CNI score and renal the lowest (Fig. 1). In 56 patients, tumor burden (computed using response criteria; refs. 1, 14) prior to therapy was correlated with the total cfDNA concentration ($r = 0.37$, $P = 0.013$) but correlated higher ($r = 0.41$, $P = 0.005$) with tumor CNI score (Supplementary Fig. S1).

Data from the discovery cohort ($n = 21$) were used to establish a model to predict outcome at the earliest possible time point. The

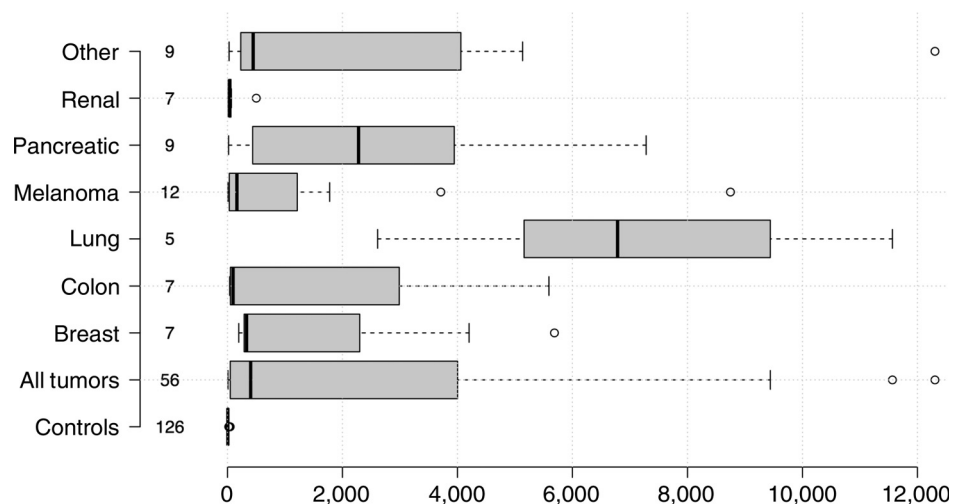
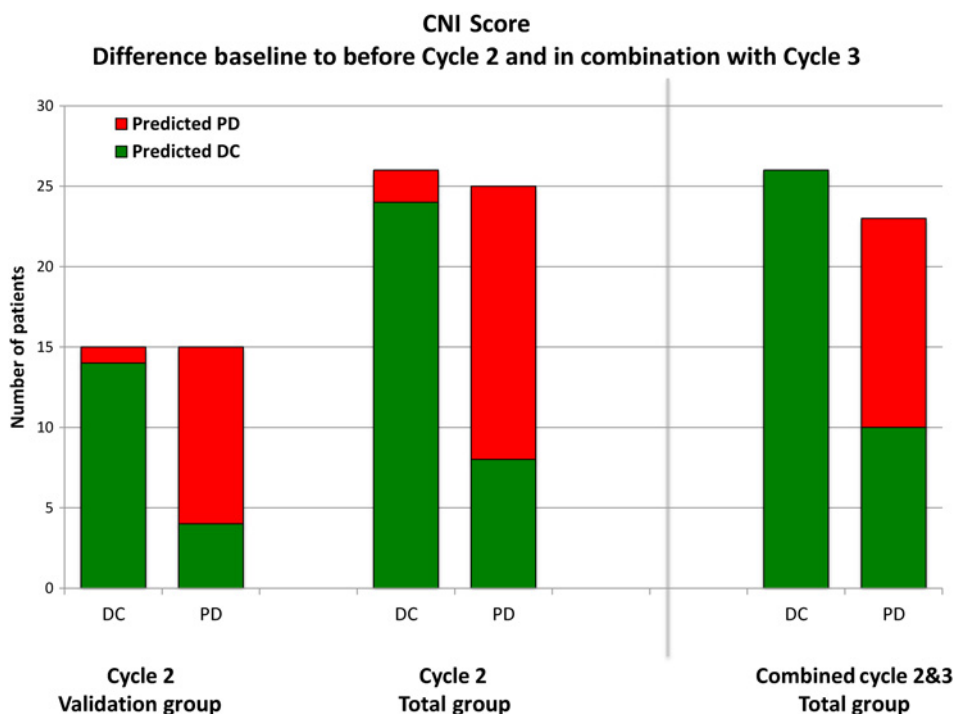


Figure 1. Univariate comparison of apparently healthy control individuals with the baseline values of all tumor patients as well as stratified by primary tumor site. Whiskers represent the 1.5-fold IQR distance from first and third quartile according to Tukey.

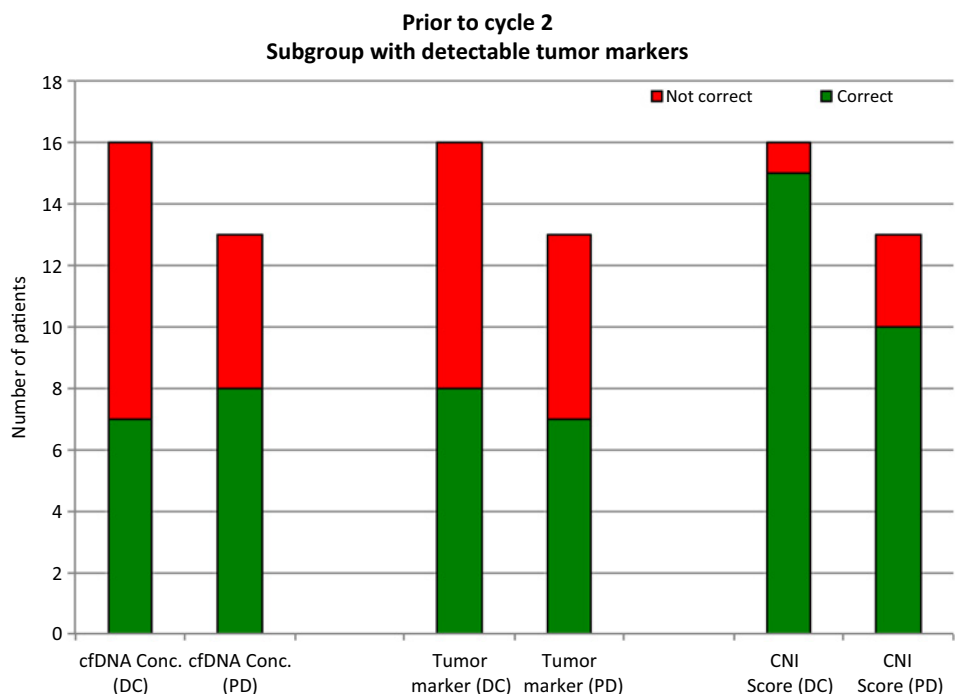
**Figure 2.**

Prediction of therapy outcome of CNI score before cycle 2 and in combination with the values determined before cycle 3. PD, progressive disease; DC, disease control (SD or CR/PR). The overall accuracy and predictive values in the validation cohort were very similar to the performance in the total study group, when values in cfDNA drawn before cycle 2 were evaluated. When both time points (prior to cycle 2 and prior to cycle 3) were used in parallel (PD is predicted when both values are indicating PD), a 100% specificity was seen (100% positive predictive value).

best and earliest prediction was possible with the CNI score before the second cycle, as well as, before the third cycle of ImmunoTx. Criteria were established using an average \pm 3SD of the difference in the CNI score between baseline and time point value of the group showing PR in imaging (Cycle 2: -174 , Cycle 3: $-2,140$), followed by a second criterion which was the absolute value of the CNI score. If the decrease of CNI score at the cycle time point did

not reach the limits above and remained above 90 (3-fold 97.5th percentile of controls), a patient was categorized as having PD.

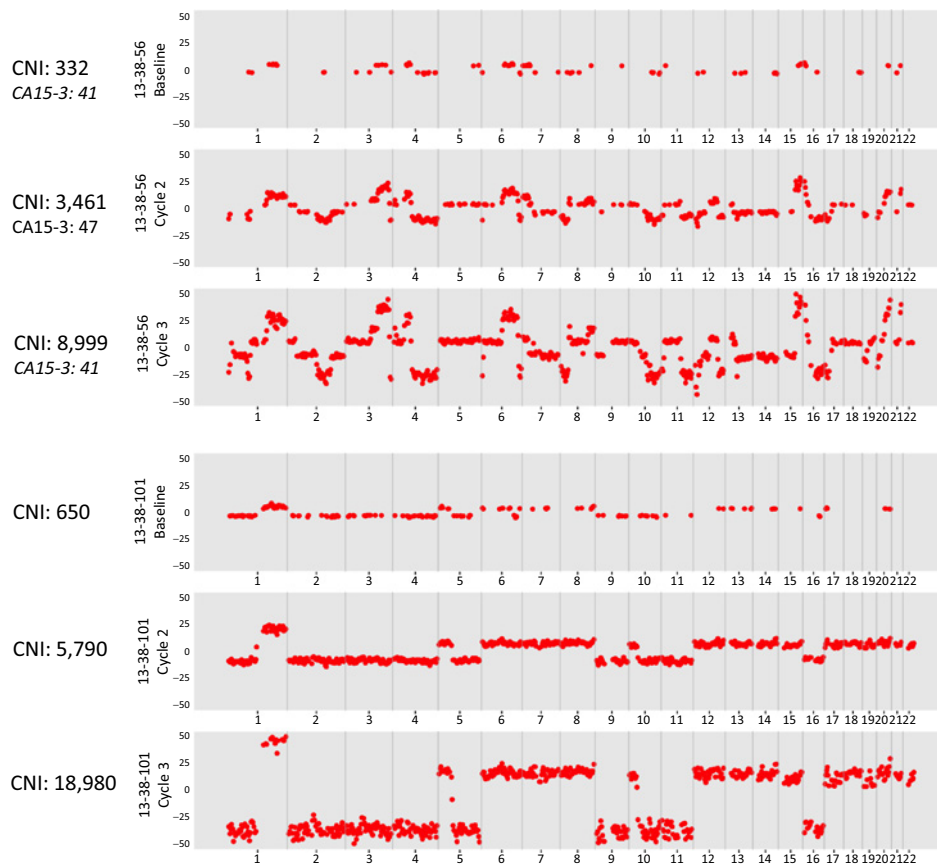
These criteria were subsequently applied to the blinded Validation cohort ($n = 30$). For samples collected prior to the second cycle in the prospective Validation cohort, we calculated an overall prediction accuracy of 83% (95% CI, 68%–93%), whereas 11 of the 15 PD patients were correctly predicted and

**Figure 3.**

Comparison of increasing/decreasing course of total cfDNA, tumor marker concentrations, and CNI-score before cycle 2 with outcome. Decreasing values under treatment are scored correct for DC and not correct for PD; increasing values are scored the opposite way. The CNI-score showed a significant distribution in to the correct groups (Fisher exact $P < 0.0002$), whereas both tumor markers ($P = 0.57$) and total cfDNA concentration ($P = 0.54$) did not.

Figure 4.

Plots showing the CNI values of significantly ($>Z = 2.84$ or $<Z = -2.84$) aberrant bins for two patients with tumor progression. The number of aberrant bins as well as the Z-score amplitude is increasing in the consecutive samplings. #13-38-56: Breast cancer with PD; #13-38-101: Melanoma with PD.



only one patient of 15 with DC was discrepant based on the predefined outcome criteria (1, 14). The positive predictive value (PPV) of the CNI score change for PD, therefore, was 92% (95% CI, 66–100%). Combination of Discovery and Validation cohorts yielded an accuracy of 80% (95% CI, 69–89%) with a PPV for PD remaining at 89% (95% CI, 70–98%). The two PD predicted patients (melanoma and lung) discrepant with DC were classified correctly by the cycle 2 criteria. When predictions were based on a combination of cycle 2 and cycle 3 criteria, a PPV for PD of 100% (95% CI, 79–100%) was obtained where an accuracy of 80% (95% CI, 68–89%) was retained. The results are summarized in Fig. 2.

TuM biomarkers (CEA, CA19-9, CA15-3, CA-125, and/or β HCG) were available in 29 patients prior to and during therapy at the identical blood raw time points as cfDNA. To assess the correlation with outcome, the direction of the concentration under treatment was estimated (rising or falling prior to cycle 2 and cycle 3 time points) and compared with response outcome. There was no consistency (nonpredictive) in the direction of TuM in 14 of 29 patients at the early pre-cycle 2 time point (accuracy: 52%, 95% CI, 35%–68%; $P = 0.57$). As shown in Fig. 3, a similar lack of predictive accuracy was found for total cfDNA (accuracy: 52%; 95% CI, 35%–68%; $P = 0.54$). In contrast, CNI score direction showed a significant correlation with outcome with only four patients categorized incorrectly (accuracy: 86%; 95% CI, 71%–95%; $P < 0.0001$). For samples before cycle 3, there was still no significant correlation for TuM (accuracy: 68%, 95% CI, 51%–82%; $P = 0.07$) or total cfDNA (accuracy: 57%, 95% CI, 40%–73%; $P > 0.27$). In contrast, correlation with CNI score

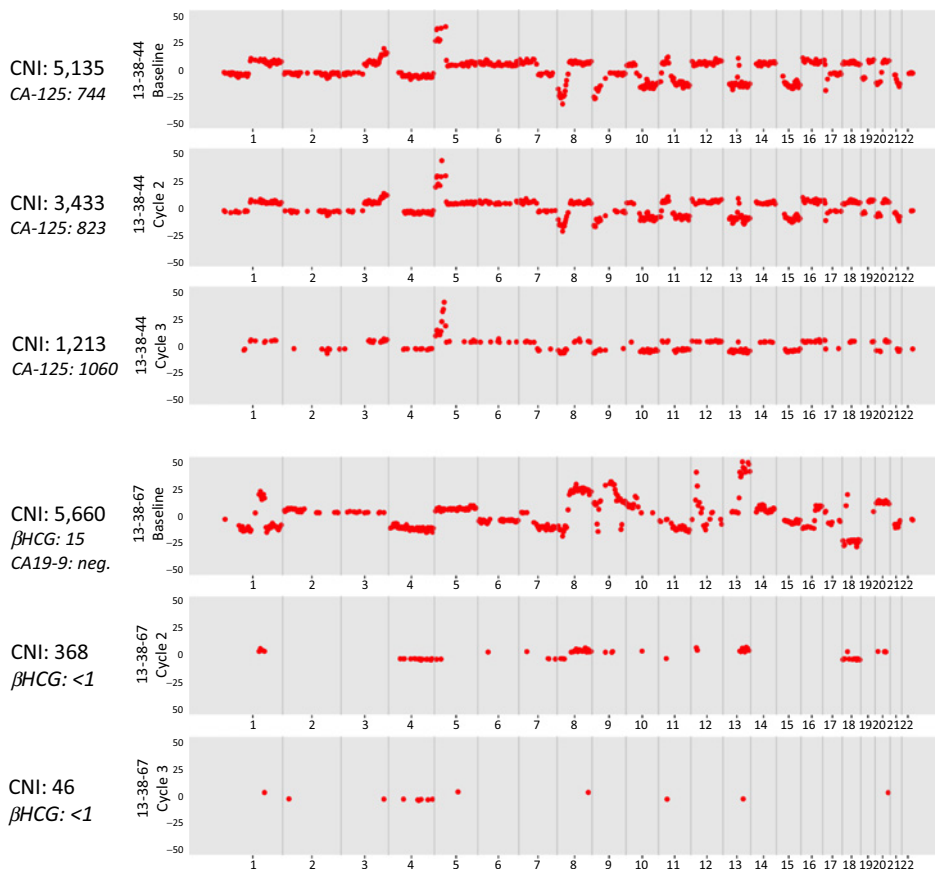
(accuracy: 82%; 95% CI, 66%–93%; $P < 0.001$) was highly significant (Supplementary Fig. S2).

Visualization of specific changes as linear genomic plots that depict all bins with significantly altered CNI values are presented for two exemplary patients with PD in Fig. 4; representative scan images of the same patients are shown in Supplementary Figs. S3 and S4. Two examples of patients with decreasing CNI scores associated with PR are illustrated in Fig. 5 and scans are shown in Supplementary Figs. S5 and S6. For easier comparison between two consecutive samplings, the "Delta Dot Plot" depicts the change of the CNI score for significantly altered bins from one time point to the next. Respective Delta Dot Plots of the four exemplary patients are shown in Supplementary Figs. S7 and S8.

A secondary objective was to investigate potential factors associated with response to ImmunoTx for which we measured the percentage of positive TSDR (TSDR%) in peripheral leukocytes. Although there was a significant decrease in the TSDR% at the last cycle compared with pretreatment ($P = 0.02$), no difference between DC and PD ($P > 0.2$) was seen. Whether this change in the circulatory Tregs is representative of a reduction of Tregs in the tumor microenvironment (reviewed in ref. 18) is unknown, but worthy of future investigation.

Discussion

Tumor cfDNA in treatment monitoring of cancer is increasingly being used for the quantification of somatic tumor-specific SNPs and the identification of escape mutations with targeted therapy.

**Figure 5.**

Plots showing the CNI values of significantly ($>Z = 2.84$ or $<Z = -2.84$) aberrant bins for two patients with tumor response. The number of aberrant bins as well as the CNI amplitude is decreasing in the consecutive samplings. #13-38-44: Ovarian cancer with PR; #13-38-67: Pancreatic cancer with PR.

The use of tissue tumor SNPs is hampered by high mutation rates in tumors and may not reflect the dominant malignant cell clone (primary/metastatic) under chemotherapy, radiotherapy, or immunotherapy. An additional complication is the frequency of SNP-positive tumors. When searching for common known driver and suppressor genes, 60% of patient samples are interrogated without a detectable somatic SNP (19).

Genomic analysis of tissue malignancies is well recapitulated by tumor cfDNA (20, 21). In contrast with a recent article evaluating cfDNA in melanoma patients receiving PD-1 inhibitor therapy (22), we did not see a clear statistically significant correlation between pretherapeutic cfDNA level and response to ImmunoTx. Furthermore, approximately 47% of the melanoma patients did not have baseline detectable cfDNA, using a SNP-based digital PCR, whereas baseline cfDNA CNI score was not elevated in only approximately 14% from our cohort of melanoma patients. As demonstrated here, over 90% of metastatic tumors are evaluable by elevated CNI scores detectable in cfDNA, which provides a real-time method for therapy monitoring with the rapid turnover of *in situ* plasma cfDNA (23). The method used herein is robust and can detect less than 1% tumor-derived cfDNA in plasma, which is demonstrated by the values determined in an artificial control with 1% tumor DNA (see Materials and Methods). The 5th percentile (CNI score = 49) is about 2-fold higher than the 95th percentile of healthy individuals (CNI score = 25). This makes such a test relatively universally applicable. The key finding is the ability of early prediction of outcome of ImmunoTx 3 to 4 weeks after the first dose, across a variety of cancer types and different regimens of ImmunoTx. If the measured CNI score does

not decrease substantially, the chance of progression is over 90% in our validation group. We have seen similar effects for cytotoxic chemotherapy (24). These data justify prospective studies in specific cancer cohorts with defined approved treatment to validate the apparent universality of the CNI method for quantifying chromosomal instability.

The evaluation of efficacy of ImmunoTx is still a critical endeavor and has several constraints, such as pseudo-tumor progression from delayed imaging and intratumor inflammatory responses. Given the high costs of these therapies, early guidance as to whether a patient might benefit has not only a potential financial impact, but also a medical advantage to change therapy sooner, if it is not going to be effective or the therapy might lead to hyperprogression (4). In our series, we observed six cases of hyperprogression (examples are given in Fig. 4 and Supplementary Figs. S3 and S4), and five of these cases were predicted to have PD by the CNI score at a significantly earlier time (~ 6 to 9 weeks) than with routine practice of imaging assessment. We also observed one case (no. 13-38-44) of pseudo-tumor progression (2, 3) by imaging (see Fig. 5 and Supplementary Fig. S5) with an initial increase in CA-125, and at each CNI time point, there was a decline consistent with prediction for a response to ImmunoTx.

The decrease in circulating Treg could be interpreted as a general activation sign of the immune system, whereas other factors such as the tumor microenvironment and dendritic cell activation play critical roles (25) for efficacy. The value of Treg content determination in the tumor microenvironment as a predictor of ImmunoTx efficacy cannot be assessed by this investigation, which was focused on blood-based analytics.

One primary aim of this study was to determine whether there are specific tumors that are less suited for this copy number–dependent analysis. Overall, renal cancers had lower CNI scores, which might be due to location, vasculature, or immune phenotype and merits further investigation. All other solid tumor types investigated in this work showed a uniform behavior for response outcome with ImmunoTx. Limitations due to low release of tumor DNA into the bloodstream will counteract any cfDNA method and most likely any blood-born analytic. Also, tumors with only few or copy number neutral genomic alterations will be challenging, although such seem rare, based on the data presented herein.

The other primary objective was to compare the CNI score with conventional tumor markers. Interestingly and noteworthy, the biochemical TuM was found to be very unreliable under therapy, when their direction was compared with outcome. This is well in line with the overall cautious recommendations made for their use in therapy monitoring (26, 27). The comparison revealed that CNI scores in plasma outperformed TuM substantially.

Increased total cfDNA concentrations have been shown in cancer (reviewed in ref. 28), and proposed for therapy monitoring (29–31). We could not establish any substantial predictive value for outcome, which is consistent with other recent literature (32, 33).

In summary, the evaluation of CNI can serve as an early indicator of response to ImmunoTx. The method is reproducible, robust, can be accomplished in one working week, and appears suitable for use in high complexity clinical reference laboratories. Quantification of chromosomal instability has the potential to reduce health care costs and disease burden for cancer patients. Larger studies focused on specific cancers, such as pancreatic and lung, are planned to establish cut-off values for specific tumors and therapies, which are needed for wider clinical use of this pharmacodynamic monitoring of systemic cancer therapies using cfDNA. Furthermore, evaluating clinical drug development studies may significantly decrease the burdensome costs of phase I

clinical trials by allowing intratrial adjustment to drug doses and scheduling not possible with imaging protocols.

Disclosure of Potential Conflicts of Interest

G.J. Weiss reports receiving speakers bureau honoraria from Medscape, Merck, Novartis, and Pfizer, and is a consultant/advisory board member for Paradigm and Viomics. J. Beck, K. Bornemann-Kolatzki, H. Urnovitz, and E. Schütz are employees of and hold ownership interest in Chronix Biomedical. W.M. Mitchell holds ownership interest in Chronix Biomedical and is on the Board of Directors of Chronix Biomedical. D.P. Braun is on the Board of Directors of Celsion Pharma. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G.J. Weiss, H. Barilla, R. Cubello, W. Quan, Jr., A. Sangal, V. Khemka, J. Waypa

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G.J. Weiss, J. Beck, D.P. Braun, K. Bornemann-Kolatzki, A. Sangal, W.M. Mitchell, H. Urnovitz, E. Schütz

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