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Analytical Validation of a Hybrid Capture—Based Next-Generation Sequencing Clinical Assay for Genomic Profiling of Cell-Free Circulating Tumor DNA

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Genomic profiling of circulating tumor DNA derived from cell-free DNA (cfDNA) in blood can provide a noninvasive method for detecting genomic biomarkers to guide clinical decision making for cancer patients. We developed a hybrid capture—based next-generation sequencing assay for genomic profiling of circulating tumor DNA from blood (FoundationACT). High-sequencing coverage and molecular barcode—based error detection enabled accurate detection of genomic alterations, including short variants (base substitutions, short insertions/deletions) and genomic re-arrangements at low allele frequencies (AFs), and copy number amplifications. Analytical validation was performed on 2666 reference alterations. The assay achieved >99% overall sensitivity (95% CI, 99.1%—99.4%) for short variants at AF >0.5%, >95% sensitivity (95% CI, 94.2%—95.7%) for AF 0.25% to 0.5%, and 70% sensitivity (95% CI, 68.2%—71.5%) for AF 0.125% to 0.25%. No false positives were detected in 62 samples from healthy volunteers. Genomic alterations detected by FoundationACT demonstrated high concordance with orthogonal assays run on the same clinical cfDNA samples. In 860 routine clinical FoundationACT cases, genomic alterations were detected in cfDNA at comparable frequencies to tissue; for the subset of cases with temporally matched tissue and blood samples, 75% of genomic alterations and 83% of short variant mutations detected in tissue were also detected in cfDNA. On the basis of analytical validation results, FoundationACT has been approved for use in our Clinical Laboratory Improvement Amendments—certified/College of American Pathologists—accredited/New York State—approved laboratory. *(J Mol Diagn 2018, 20: 686—702; https://doi.org/10.1016/j.jmoldx.2018.05.004)*

Sequencing of cancer genomes has yielded insights into the genomic alterations that drive different cancer types, and has led to the development of numerous therapies that target genetic vulnerabilities of tumors. With the increasing number of genomic alterations that are either predictive biomarkers for approved targeted therapies or used as inclusion criteria for genomically matched clinical trials, comprehensive genomic profiling of tissue samples using

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next-generation sequencing (NGS) to evaluate hundreds of cancer-related genes has transitioned from the research setting into an important tool for routine clinical management of patients with cancer.[1–4]

Several tumor tissue–based companion diagnostic NGS assays have been approved by the US Food and Drug Administration for the identification of genomic biomarkers to guide treatment with targeted therapies,[4–6] and on the basis of extensive studies using tissue samples to define the genomic landscape of cancer, tissue-based testing represents the gold standard for genomic profiling.[2,3] However, in some cases, obtaining a tissue sample may not be possible because of inaccessibility of the tumor, risk of complications from the tissue biopsy, or insufficient tissue.[7] Because approximately 80% of metastatic solid tumors release cell-free circulating tumor DNA (ctDNA) into the circulation,[8] sequencing of cell-free DNA (cfDNA) from blood could provide an alternative method for identifying genomic changes in the tumor tissue. Recently, a plasma-based PCR test for epidermal growth factor receptor (*EGFR*) mutations in patients with non–small-cell lung cancer (NSCLC) was US Food and Drug Administration approved as a companion diagnostic for EGFR tyrosine kinase inhibitors (TKIs).[9] Therefore, ctDNA provides an opportunity to perform noninvasive blood-based genomic profiling should a tissue sample be unavailable.

Blood-based testing of ctDNA offers the advantage of simple and rapid sample collection and may be particularly suited to serial genomic profiling for identifying resistance mutations and monitoring disease burden. An understanding of acquired genomic alterations that mediate resistance to first-line targeted therapies has led to the development of subsequent targeted therapies that are designed to be active against resistance mutations, such as the EGFR TKI osimertinib for the *EGFR* T790M mutation in NSCLC[10]; serial genomic profiling assessments of ctDNA may provide a convenient method to monitor emergence of resistant clones and identify mechanisms of resistance to guide selection of later-line targeted therapies. Furthermore, because the abundance of ctDNA in blood is associated with tumor size, serial genomic profiling of ctDNA may be used for longitudinal assessment of disease burden to detect minimal residual disease, identify relapse, and monitor response to therapy.[11–13]

The development of NGS-based gene panels to sequence ctDNA has allowed blood-based genomic profiling of early- and late-stage cancers.[11,14–17] Because ctDNA typically comprises a small fraction of the total cfDNA, sensitive techniques are required to detect sequence alterations in ctDNA that frequently exist at low abundance.[15] In this study, we describe the development and analytical validation of a hybrid capture–based NGS clinical assay of ctDNA in blood (FoundationACT). High-sequencing coverage and molecular barcode–based error detection allowed for accurate and sensitive detection of genomic alterations in ctDNA, including base substitutions, short insertions/deletions (indels), and re-arrangements/fusions at low allele frequencies (AFs), as well as copy number amplifications (CNAs).

Rigorous validation studies are required to demonstrate robust analytical performance. Therefore, extensive validation was performed by: i) constructing a validation set of 2666 genomic alterations encompassing all tested alteration types across the spectrum of genes targeted by the assay; ii) assessing performance across a broad range of allele frequencies; iii) validating performance at sequencing coverages that are reflective of the range routinely achieved in clinical samples; iv) demonstrating, using clinical cfDNA samples, that the results of the assay are concordant with orthogonal methods; and v) establishing that the genomic profiling results from the FoundationACT assay are consistent with tissue-based genomic profiling. On the basis of the analytical validation studies, FoundationACT has been approved for use in our Clinical Laboratory Improvement Amendments (CLIA)–certified, College of American Pathologists (CAP)–accredited, New York (NY) State–approved laboratory.

Materials and Methods

Whole Blood Collection, Plasma Isolation, and cfDNA Extraction

Clinical samples for analytical validation and comparison with orthogonal approaches were received as whole blood or archival frozen plasma stored at −80°C. For blood samples, 16 to 20 mL peripheral blood was collected in Cell Free DNA Blood Collection Tubes (Roche, Pleasanton, CA) or Cell-Free DNA BCT tubes (Streck Inc., La Vista, NE). To isolate plasma: i) whole blood was centrifuged at 1600 × *g* for 20 minutes at room temperature, ii) supernatant was collected and centrifuged at 16,000 × *g* for 20 minutes at 4°C, and iii) supernatant was collected as plasma that underwent cfDNA extraction. Plasma was treated with proteinase K for 20 minutes at 60°C and mixed with 1.25 × volume of cfDNA binding solution (Thermo Fisher Scientific, Waltham, MA) and 500 ng/mL of paramagnetic MyOne SILANE beads (Thermo Fisher Scientific). Beads were washed twice with cfDNA wash solution (Thermo Fisher Scientific) and twice with 80% ethanol, and they were eluted in cfDNA elution solution (Thermo Fisher Scientific). cfDNA concentration was determined using the D1000 ScreenTape assay on the 4200 TapeStation (Agilent Technologies, Santa Clara, CA). cfDNA (20 to 100 ng) was used for library construction.

Library Construction

Library construction was performed on the Bravo Benchbot (Agilent Technologies) automation system with NEBNext reagents (NEB, Ipswich, MA) containing mixes for end repair, dA addition, and ligation using the with-bead protocol to maximize library yield and complexity. A set of 12

fragment-level indexed adaptors with variable 6-bp DNA barcodes were ligated randomly onto both ends of each input duplex cfDNA fragment. Ligated sequencing libraries were PCR amplified with a universal PCR primer and an indexed PCR primer with a high-fidelity polymerase (Kapa Biosystems, Wilmington, MA) for 10 cycles, 1.8 × Solid Phase Reversible Immobilization purified, and quantified by PicoGreen (Invitrogen, Carlsbad, CA). Samples yielding 500 to 2000 ng of sequencing library proceeded to hybrid capture.

Panel Design, Hybrid Capture, and Sequencing

Solution hybridization was performed using a >50-fold molar excess of a pool of 2695 individually synthesized 120-bp 5′-biotinylated single-stranded DNA oligonucleotide baits (assay baitset version CF2; Integrated DNA Technology, Coralville, IA). The baitset targeted 140,419 bp of the human genome, including all exons of 27 genes, selected exons of an additional 33 genes (133 exons), selected introns of 6 genes frequently involved in genomic re-arrangements in cancer (12 introns), and the *TERT* promoter region that is recurrently mutated in cancer (Supplemental Table S1). The baitset also targeted 96 single-nucleotide polymorphisms (SNPs) that serve as a patient-specific signature to allow confirmation of the same subject in longitudinal test comparison. Bait design and hybridization capture were performed as described previously.[1,18] Briefly, 500 to 2000 ng of sequencing library was lyophilized with human Cot-1 DNA, sheared salmon sperm DNA, and adaptor-specific blocking oligonucleotides; resuspended in water; heat denatured at 95°C for 5 minutes; and incubated at 68°C, with the final addition of the baitset into hybridization buffer. The hybridization reaction was incubated at 68°C for 12 to 24 hours, and library-baitset duplexes were captured on paramagnetic MyOne streptavidin beads (Invitrogen). Off-target library was removed by washing once with 1 × saline-sodium citrate at 25°C and four times with 0.25 × saline-sodium citrate at 55°C. The 1 × KAPA HiFi Hotstart ReadyMix PCR mastermix (number KK2602; Kapa Biosystems) was added directly to the beads to amplify the captured library. Samples were 1.8 × Solid Phase Reversible Immobilization purified and quantified by PicoGreen (Invitrogen). Libraries were normalized to 1.05 nmol/L, pooled, and loaded onto an Illumina cBot for the template extension reaction directly on the flow cell that was loaded onto an Illumina HiSeq 4000 with 2 × 151 bp or HiSeq 2500 with 2 × 176 bp paired-end sequencing protocol (Illumina, San Diego, CA).

Process-matched normal control DNA was run in parallel with each batch of test samples to observe variation across assays and to serve as normal reference for CNA analysis. Purified normal control DNA from two individuals was obtained from the International HapMap Project (Coriell Institute, Camden, NJ), combined in a 99:1 ratio, and sheared by ultrasonication to generate approximately 200-bp fragments (Covaris, Woburn, MA).

Sequence Data Processing

Read Processing

The following steps were used to process raw sequence data: i) Reads pairs were demultiplexed by sample barcode to yield sets of reads deriving from distinct patient samples. ii) For each sample, read pairs were sorted into subsets on the basis of the fragment barcodes found at the start of each read in the pair, segregating read pairs that cannot have derived from the same fragment. iii) Read pairs within each fragment barcode pair subset were mapped to the reference genome (hg19) using BWA version 0.7.15[19] and clustered into subsets corresponding to distinct fragments. iv) Read pairs corresponding to each distinct fragment were aligned to each other (read 1 versus read 2) as well as to all other read pairs in the set to identify any experimentally introduced sequence errors.[20] A merged complete fragment sequence was generated when possible (typically when the fragment size was <250 bp), whereas a paired representation was retained for larger fragments. Any errors identified were marked as such.

Variant Calling

A set of candidate variants was generated by parsing all alignments found in the consensus representation of the sequences determined for each fragment, avoiding sections marked as containing errors.[1,21] Every read in the original raw data mapping to the region of the putative variant was realigned to the candidate variant haplotypes and assessed to determine which allele was supported.[20] Read-level support within each cluster was evaluated to derive an allele assignment for the associated fragment or to determine that no such assignment could be made reliably. Given the set of allele assignments for all fragments covering the locus, a statistical model incorporating the observed redundancy level and error rate was used to determine the expected noise level for the putative variant. Given that noise level, the Poisson distribution was used to determine the probability of observing the obtained number of fragments supporting the variant. A threshold was applied for variant calling: for most variants, variant calls were made when the number of fragments unambiguously supporting the variant was greater than five; for variants at noisier loci, higher thresholds were set on the basis of the level of redundancy at the locus and the number of error-containing fragments identified.

Variant Filtering

Final variant calls were annotated for predicted protein impact and biological significance. Germline variants were removed by referencing dbSNP (release 135) and 1000 Genomes Project,[22] except for known pathogenic germline variants, such as certain *BRCA1/2* mutations that were considered as reportable. Reportable genomic alterations were called as known/likely functional driver alterations on the basis of presence of the specific variant in the Catalogue

of Somatic Mutations in Cancer[23] or more general knowledge about the gene affected (eg, truncations and deletions in known tumor suppressor genes or mutations that have been characterized as pathogenic in the scientific literature); all other uncharacterized alterations were classified as variants of unknown significance.

Copy Number Amplification Calling

CNAs were identified by modeling both coverage variation and allele frequencies for common germline polymorphisms as a function of amplification at targeted loci, tumor ploidy, and overall tumor purity for a sample. Sequence coverage at all targets was normalized against a process-matched normal control sample and subsequently GC normalized. Measured targets were composed of exons, introns, and SNPs, which are designed to improve copy number modeling by use of allele imbalance, as previously described.[1] Thresholds were applied to the resulting CNA model on the basis of estimated tumor purity and ploidy, with the goal of reporting amplifications of at least eight copies while avoiding low-level gains.

Re-Arrangement Detection

Re-arrangements were detected by searching for chimeric alignments, where one portion of a read was aligned to a targeted gene, and the other portion was aligned to another location in the genome. Filters were applied to ensure high-quality alignments, and a minimum number of reads supporting the re-arrangement were required, as described.[1]

Reference Cell Lines, Synthetic Gene Fusions, and Clinical cfDNA Samples

For validation of base substitution and indel variant calls, purified DNA from 20 lymphoblastoid cell lines from the International Hapmap Project (HapMap cell lines) and 26 cancer cell lines were used to generate reference samples (Supplemental Table S2). Various mixtures of cell line DNA were generated, including one mixture of DNA from 20 HapMap cell lines and five mixtures of DNA derived from 26 cancer cell lines (Supplemental Table S2); cell line DNA mixtures were diluted with normal HapMap DNA (HapMap NA12878) at varying ratios to generate reference samples for validation. Mixtures were generated by pooling in equal parts using a Biomek NX (Beckman Coulter, Pasadena, CA) and making dilutions with normal HapMap DNA; final expected mutant allele frequencies (MAFs) were calculated on the actual mixing ratios using a linear regression of SNP alternate AFs in the pools (Supplemental Table S3).

For re-arrangement validation, reference samples were generated from two mixtures of DNA derived from cancer cell lines (Supplemental Tables S4 and S5) that were diluted with normal HapMap DNA at varying ratios and five synthetic 1-kbp dsDNA gBlock gene fusion constructs (Integrated DNA Technology) spiked in to fusion-negative cfDNA isolated from clinical samples at varying ratios (Supplemental Tables S4 and S5).

For CNA validation, DNA from three tumor-normal paired cell lines was used (Supplemental Table S6); DNA from each cell line was individually diluted into its paired normal DNA at varying ratios to generate reference samples. A further 29 reference clinical cfDNA samples with confirmed amplification by orthogonal assays were included in the analysis (Supplemental Table S6).

Reference DNA samples were sheared to cfDNA-sized fragments (approximately 200 bp) by ultrasonication (Covaris), and 100 ng DNA was analyzed by the FoundationACT assay.

Genomic Profiling to Determine Reference Variants in Cell Lines

Cell line DNA samples were sequenced individually using the FoundationOne NGS assay[1] to determine the reference variants present, including base substitutions and indels in dbSNP for HapMap cell lines and base substitutions, indels, re-arrangements, and CNAs for cancer cell lines. The expected MAF for each variant in the pooled reference samples was calculated on the basis of the allele frequency in the original cell line and the composition/dilution of the reference DNA mixtures (Supplemental Tables S3 and S5).

Samples from Healthy Individuals

cfDNA was extracted from blood samples from volunteers, aged 18 to 65 years, who all self-reported as healthy without history of cancer (Research Blood Components, Boston, MA).

Comparison with Orthogonal Assays Used for Clinical Samples

For concordance analyses, clinical cfDNA samples were processed by FoundationACT, and select genomic alterations were also evaluated using orthogonal confirmatory assays, including droplet digital PCR (ddPCR), beads, emulsions, amplification, and magnetics (BEAMing), FoundationOne NGS, and breakpoint PCR, as outlined below. All primers/probes used for ddPCR and breakpoint PCR assays are listed in Supplemental Table S7.

ddPCR

For select base substitutions and indels, probes and primers were either predesigned PrimePCR Mutation Assays (Bio-Rad, Hercules, CA) or custom synthesized (Integrated DNA Technology) and designed according to the ddPCR Applications Guide (Bio-Rad). Dual-quenched probes were synthesized with 5′ HEX or FAM reporter, an internal ZEN quencher, and an Iowa Black FQ 3′ quencher. For CNAs, probes and primers were predesigned PrimePCR Copy Number Variation Assays (Bio-Rad). Each reaction contained

the following: 1 × ddPCR Supermix for Probes (no dUTP; number 186-3026; Bio-Rad), 250 nmol/L of each probe, 450 nmol/L (for base substitutions/indels) or 900 nmol/L (for CNAs) of each primer, and 30 ng of cfDNA library in a 20-μL reaction volume. Emulsion PCR amplifications were performed in the C1000 Touch Thermal Cycler (Bio-Rad), as follows: 1 cycle of 95°C for 10 minutes, 40 cycles of 94°C for 30 seconds and 55°C for 1 minute (base substitutions/indels) or 60°C for 1 minute (CNAs), and 1 cycle of 98°C for 10 minutes. Droplets were read with the QX200 droplet reader (Bio-Rad). QuantaSoft version 1.7.4 (Bio-Rad) was used to calculate fractional abundance of base substitutions/indels and set thresholds for CNA calling. Positive CNAs were identified as greater than the average ddPCR ratio from wild-type samples plus 3 SDs.

BEAMing Digital PCR

Plasma samples from a phase 2 study in hormone receptor−positive, human epidermal growth factor receptor 2 (HER2)−negative metastatic breast cancer (NCT01740336)[24] were sent to an external laboratory (Sysmex-Inostics, Baltimore, MD) to perform BEAMing; *ESR1* E380Q, Y537C/S/N, and D538G base substitutions were assessed for concordance.

Hybrid Capture−Based NGS with FoundationOne

Extracted cfDNA (≥50 ng) was submitted for processing to the clinical laboratory at Foundation Medicine (Cambridge, MA) to be analyzed on the FoundationOne NGS-based clinical cancer test[1] that includes all targeted territory of the FoundationACT assay baitset.

Breakpoint PCR

Each reaction contained the following: 1 × PCR supermix, 450 nmol/L of each primer, and 30 ng of cfDNA library in a 20-μL reaction volume. PCR amplifications were performed in the C1000 Touch Thermal Cycler as follows: 1 cycle of 95°C for 10 minutes, 40 cycles of 94°C for 30 seconds and 55°C for 1 minute, and 1 cycle of 98°C for 10 minutes. All samples, including no template controls, were run in triplicate. PCRs were analyzed with the Agilent TapeStation D1000 assay (Agilent Technologies) for expected product size in the library and positive control with no amplification in the wild-type DNA and no template control. Breakpoint PCR primers were as follows: *ROS1* re-arrangement (forward, 5′-CATGACTGTCTTGGGCAATG-3′; reverse, 5′-CCCAAATGAGGCAACTGTCTA-3′), *SMO* re-arrangement (forward, 5′-GCAGATGTGCAAATATCTGGT-3′; reverse, 5′-CAGGAAGCCAAAAATGCCTG-3′), *MYC* re-arrangement (forward, 5′-CGTTAGCTTCACCAACAGGA-3′; reverse, 5′-TCATTTCCCACTTGCCACAT-3′), *VEGFA* re-arrangement (forward, 5′-AGGAAGAGTAGCTCGCCG-3′; reverse, 5′-ACAGCTGCTTTCTCACAGAG-3′), *KIF5B-RET* (forward, 5′-TCACCAAACCCAATATCACCT-3′; reverse, 5′-ACTGCTCCGGATGCCTTC-3′), *EML4-ALK* number 1 (forward, 5′-CAGGCTGGAATGCTGTAGAA-3′; reverse, 5′-TAAGAGCTGGTTGGGACCAC-3′), *EML4-ALK* number 2 (forward, 5′-GCCAGAAATTGTTTGAAGTGC-3′; reverse, 5′-CCTGATCAGCCAGGAGGATA-3′), *EML4-ALK* number 3 (forward, 5′-AGGCTGCATGGAATCTGAA-3′; reverse, 5′-GTAGGGCAGCTTCAGTGCAA-3′), *EML4-ALK* number 4 (forward, 5′-TGTTTTCACCGAAATGTGGA-3′; reverse, 5′-AGGAATTGGCCTGCCTTAGT-3′), *EML4-ALK* number 5 (forward, 5′-CTGGAGGCAGGGAGGAATA-3′; reverse, 5′-TACATAGGGTGGGAGCCAAA-3′), *EML4-ALK* number 6 (forward, 5′-CAGGCACCATGTATAAAATTGCT-3′; reverse, 5′-ACAGAGTTGGAGAAGAGCCA-3′), *EML4-ALK* number 7 (forward, 5′-TCAGGGGCGCTAATGAACA-3′; reverse, 5′-TGCTCAGCTTGTACTCAGGG-3′), *EML4-ALK* number 8 (forward, 5′-ACACCTGAGATAACTGTCCCA-3′; reverse, 5′-TCTGGAGCCAAAGTCAGTCA-3′), and *EML4-ALK* number 9 (forward, 5′-TACGTGCTCGGCAATTTACA-3′; reverse, 5′-GGGACTGATCAAAGCAGAA-3′).

Calculation of Performance Statistics

For sensitivity analysis, the reference alteration set was defined on the basis of FoundationOne NGS results from component cell lines analyzed individually. Each variant found in any cell line at >15% allele frequency was included in the reference set (a conservative threshold chosen to ensure a high-quality allele frequency estimate). Expected allele frequencies for all variants in the cell line mixes were determined on the basis of mixing ratios: mixing ratios were adjusted to account for variability in the mixing process and calculated on the basis of the observed allele frequency of variants that were unique to each component cell line in the mixture. All on-target variants from reference samples with an expected MAF ≥0.125% were assigned either a true positive (TP) if detected or false negative (FN) if not detected (Supplemental Tables S3 and S5). Sensitivity was calculated as follows: TP/(TP + FN).

For positive predictive value (PPV) analysis, each called variant was classified as a TP if a matching alteration was detected in the reference sample or as a false positive (FP) if a matching alteration was not detected. PPV was calculated as follows: TP/(TP + FP).

One variant (*ERBB2* P232T chromosome 17:37866389 C>A) that was observed by FoundationACT at low allele frequency was confirmed to be present in the reference samples by ddPCR and was excluded as an FP from analysis. Calls made at the top dilutions within a dilution series at low allele frequency were excluded from the analysis as unconfirmed, but were likely true positives as any variant at <0.5% allele frequency in the top-dilution cell line mixture could reasonably have been present at an allele frequency of <15% in a component cell line.

The unique coverage obtained for the validation experiments was biased toward the top end of the range of coverages observed for clinical samples (Supplemental Figure S1A). To determine performance measures that match the full spectrum

of coverage of routine clinical samples, including those at the lower bounds of coverage, the validation data sets were downsampled to lower levels of coverage (Supplemental Figure S1B). For clinical samples, lower fragment-level coverage is accompanied by increased redundancy (read pairs per fragment). It is not possible to simulate increased redundancy or the higher quality of data that comes with it, meaning that the sampled data set represents a lower limit of performance. To prevent additional loss of redundancy in the simulation, the sampling was performed at the fragment level, retaining all reads associated with each selected fragment. The overall coverage distribution that we observe in clinical practice was approximated well by an equally weighted combination of samplings to 40%, 50%, 60%, 70%, 80%, 90%, and 100% of the initial experimental data set (Supplemental Figure S1). The final reported performance statistics reflect this averaged sampled data set.

Prospective Clinical Genomic Profiling Results of FoundationACT

Approval for this study, including a waiver of informed consent and a Health Insurance Portability and Accountability Act of 1996 waiver of authorization, was obtained from the Western Institutional Review Board (protocol 20152817). Samples (16 to 20 mL whole blood) were submitted by clinicians for genomic profiling in the course of routine clinical care and processed in our CLIA-certified, CAP-accredited, NY State–approved laboratory using the FoundationACT assay, as described above. Data are presented from 884 consecutive clinical samples analyzed by FoundationACT. For the most common cancer types sequenced, the frequency of genomic alterations observed by genomic profiling of cfDNA in this study were compared with the corresponding frequency in the Foundation Medicine database of genomic profiling results from tissue samples sequenced using the FoundationOne assay,[2] containing >10,000 cases of NSCLC, breast cancer, and colorectal cancer and >2000 cases of prostate cancer. For 36 patients, temporally matched tissue samples were sequenced using the FoundationOne assay and assessed for concordance with blood samples analyzed by FoundationACT; concordance analysis was limited to reportable genomic alterations that are covered by both assays.

Results

Hybrid Capture–Based NGS Assay for Genomic Profiling of ctDNA from Blood

The FoundationACT assay was developed to identify genomic alterations from ctDNA in the blood of patients with cancer. A summary of the assay workflow is outlined in Figure 1. In brief, ≥20 ng of cfDNA was extracted from plasma and underwent library construction, where input cfDNA fragments were tagged with molecular fragment barcodes. Sequencing libraries underwent hybridization capture using a custom gene panel and were sequenced to generate >50 million read pairs of raw data, which typically correspond to a raw on-target coverage of >25,000×. Fragment barcode–based error detection enabled detection of genomic alterations, including short variant mutations (base substitutions and indels) and re-arrangements at low AFs, as well as CNAs.

Validation Approach

To estimate the accuracy of the test, reference samples with defined variants in a diversity of assayed genes were generated using DNA from normal HapMap cell lines, cancer cell lines, synthetic DNA constructs, and clinical cfDNA samples; 100 ng of each reference sample was analyzed by FoundationACT. Sensitivity and PPV were

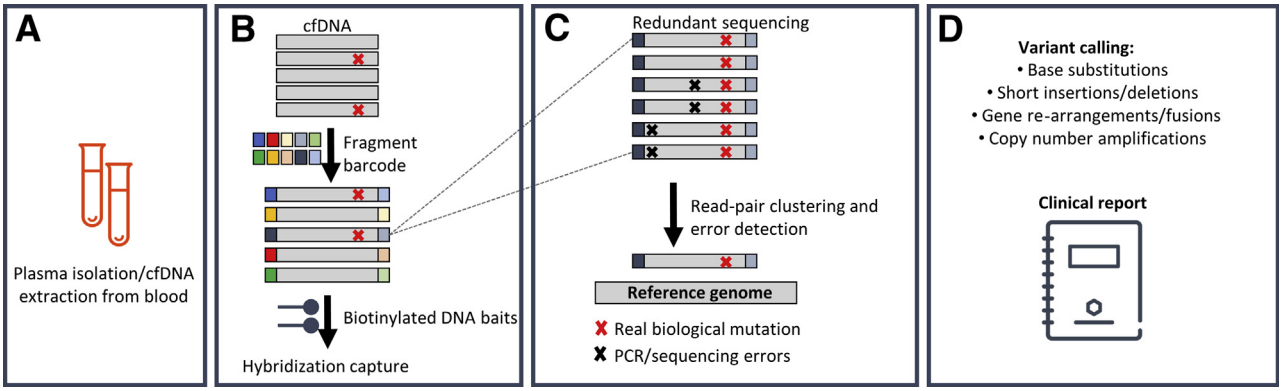


Figure 1 ctDNA genomic profiling assay workflow and fragment molecular barcode–based sequencing and error detection approach. **A:** Peripheral whole blood (16 to 20 mL) is collected in cfDNA collection tubes, plasma is isolated, and cfDNA is extracted. **B:** cfDNA (20 to 100 ng) undergoes library construction, tagging with fragment barcodes, library amplification, and hybridization capture. **C:** Sequencing is performed using the Illumina HiSeq 4000 platform (2 × 151 bp paired-end sequencing) to generate 50 to 100 million read pairs. Fragment barcodes are used to identify multiple reads originating from the same unique input cfDNA fragment for subsequent error detection. **D:** Base substitutions, insertions/deletions, gene re-arrangements, and copy number amplification are called, considering detected errors. Benign germline variants are filtered (dbSNP and 1000 Genomes Project). Driver alterations are called as known and clinically annotated to highlight potential matching approved targeted therapies and clinical trials.

determined by comparing the variants detected by the FoundationACT assay with expected variants from the reference samples. To evaluate assay performance across the range of cfDNA input mass (20 to 100 ng) used for the assay in clinical samples, *in silico* down-sampling was performed (random selection of subsets of fragments) of data from reference samples to generate a sampled data set that simulated coverage observed in lower cfDNA input mass samples (Supplemental Figure S1). Results presented herein represent the aggregate data from multiple samplings of the primary experimental data set to generate a coverage profile similar to that obtained in routine clinical samples (Supplemental Figure S1). A summary of the analytical validation results is presented in Table 1.

To evaluate the performance of the assay in the clinical setting, the concordance between alterations detected by FoundationACT in clinical cfDNA samples with multiple orthogonal approaches run on the same samples, including a validated clinical NGS assay (FoundationOne),[1] ddPCR, BEAMing, and breakpoint PCR, was examined. The results from 884 consecutive clinical cases that were prospectively sequenced using FoundationACT in our CLIA-certified/CAP-accredited/NY State–approved laboratory are also presented; these results were compared with tissue-based genomic profiling. The results of these analyses are described in the proceeding sections.

Analytical Validation of the FoundationACT Assay

To evaluate analytical performance of calling base substitutions, indels, and re-arrangements, reference DNA samples derived from cell lines or synthetic DNA constructs with defined alterations at a broad range of MAFs biased toward a low target level of detection were generated (Figure 2, A–C).

Base Substitutions and Indels

Reference samples for base substitutions and indels consisted of DNA mixtures derived from 46 reference cell lines (Supplemental Table S2). A total of 2399 expected base substitutions were evaluated (median MAF, 0.80%), with 1544 at MAF ≥0.5%, 440 at MAF 0.25% to 0.5%, and 415 at MAF 0.125% to 0.25% (Figure 2A and Supplemental Table S3). For base substitutions with an expected MAF ≥0.5%, the assay achieved >99% sensitivity (95% CI, 99.1%–99.4%) with 100% PPV (95% CI, >99.9%–100%). At an expected MAF 0.25% to 0.5%, the assay achieved >95% sensitivity (95% CI, 94.9%–96.4%) with >100% PPV (95% CI, 99.8%–100%). At an expected MAF 0.125% to 0.25%, the assay achieved 70% sensitivity (95% CI, 68.3%–71.6%) with >99% PPV (95% CI, 99.8%–100%) (Table 1 and Supplemental Table S3). Detected base substitutions demonstrated high correlation with expected MAFs on the basis of the composition of the mixtures (Pearson correlation $r = 0.971$) (Figure 2D and Supplemental Table S3).

The reference mixtures also contained a total of 183 expected indels (of length in the range of 1 to 40 bp; median MAF, 0.84%), with 115 at MAF ≥0.5%, 34 at MAF 0.25% to 0.5%, and 34 at MAF 0.125% to 0.25% (Figure 2B and Supplemental Table S3). For indels, the assay achieved 100% PPV, with no FPs detected across the range of MAFs evaluated. Sensitivity was >98% (95% CI, 97.3%–99.2%) at an expected MAF ≥0.5%, >85% (95% CI, 81.4%–90.5%) in the MAF 0.25% to 0.5% range, and >65% (95% CI, 62.1%–74.3%) in the MAF 0.125% to 0.25% range (Table 1 and Supplemental Table S3). Detected indels also demonstrated high correlation with expected MAFs (Pearson correlation $r = 0.866$) (Figure 2E and Supplemental Table S3).

Table 1 Summary of the Analytical Validation Results

Variable	MAF, %	Sensitivity, %		PPV, %	
		Value	95% CI	Value	95% CI
Base substitutions	≥0.5	99.3	99.1–99.4	100	>99.9–100
	0.25–0.5	95.7	94.9–96.4	100	99.8–100
	0.125–0.25	70.0	68.3–71.6	99.9	99.8–100
Indels	≥0.5	98.5	97.3–99.2	100	99.4–100
	0.25–0.5	86.6	81.4–90.5	100	97.8–100
	0.125–0.25	68.5	62.1–74.3	100	97.1–100
Re-arrangements	≥0.5	100	77.1–100	100	77.1–100
	0.25–0.5	100	56.1–100	100	56.1–100
	0.125–0.25	80.0	29.9–99.0	100	39.6–100
CNAs*	≥20% ctDNA fraction	95.3	82.9–99.%	97.6	85.9–99.9
	<20% ctDNA fraction	Varies depending on amplitude of CNA and ctDNA fraction			
Reproducibility		100%, Interbatch precision 100%, Intrabatch precision			

*For genes with four or more targets (Supplemental Table S1).

CNA, copy number amplification; Indel, insertion/deletion; MAF, mutant allele frequency.

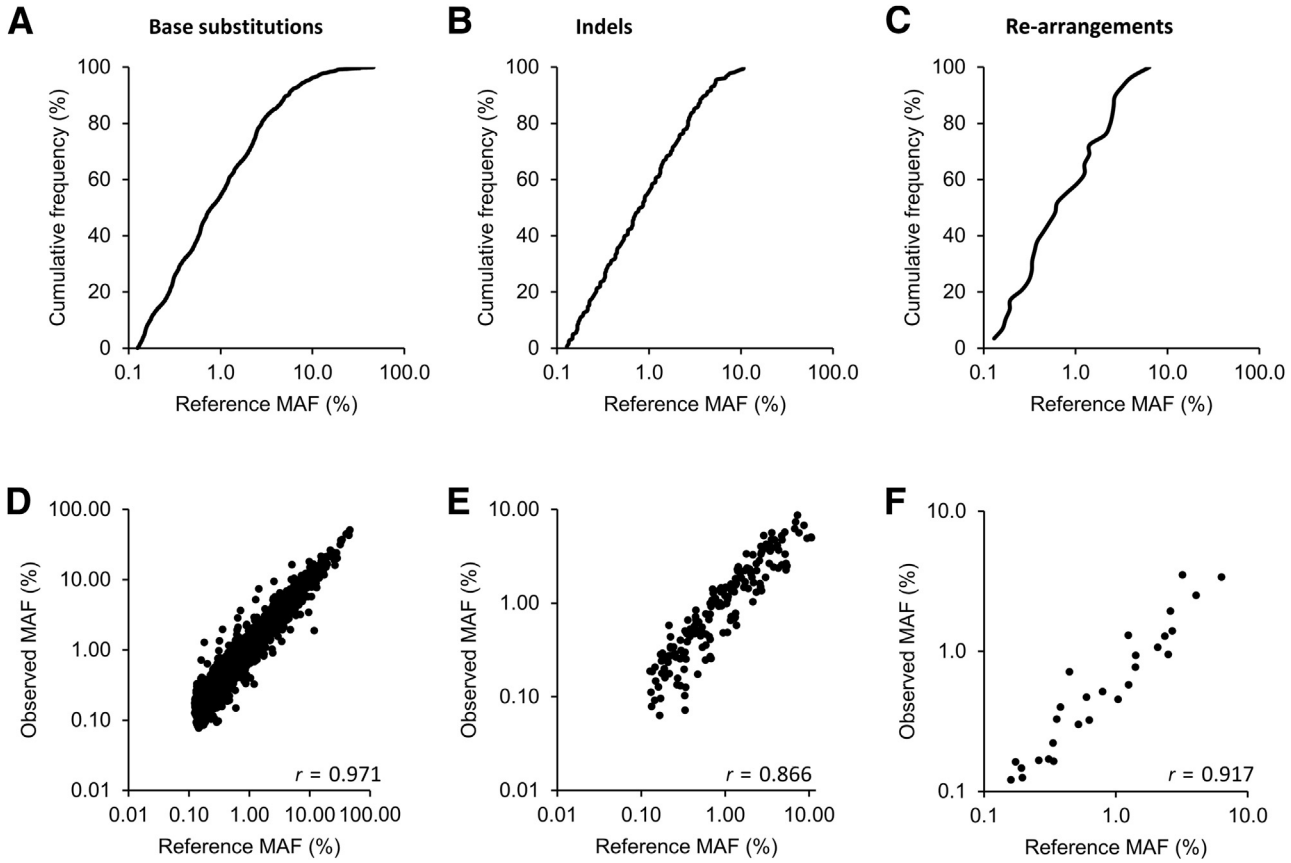


Figure 2 Analytical validation of the assay for base substitutions, insertions/deletions (indels), re-arrangements, and amplifications. **A**–**C:** Cumulative frequency of expected mutant allele frequencies (MAFs) from reference HapMap and cancer cell line samples or synthetic gene fusion constructs for base substitutions (**A**), indels (**B**), and re-arrangements (**C**). **D**–**F:** Observed MAFs of detected genomic alterations correlate with the expected MAFs in reference samples for base substitutions (**D**), indels (**E**), and re-arrangements (**F**).

Genomic Re-Arrangements

Reference samples for genomic re-arrangements consisted of two types of samples, including mixtures of DNA from reference cancer cell lines and synthetic double-stranded DNA fusions spiked into fusion-negative clinical cfDNA samples (Supplemental Tables S4 and S5).

The reference cell line or synthetic spike-in samples contained a total of 29 expected genomic re-arrangements (median MAF, 0.63%), with 17 at MAF ≥0.5%, 7 at MAF 0.25% to 0.5%, and 5 at MAF 0.125% to 0.25% (Figure 2C and Supplemental Table S5). Sensitivity was 100% (95% CI, 77.1%–100%) at MAF ≥0.5%, 100% (95% CI, 56.1%–100%) in the MAF 0.25% to 0.5% range, and 80% (95% CI, 29.9%–99.0%) in the MAF 0.125% to 0.25% range, all with 100% PPV (Table 1 and Supplemental Table S5). Detected re-arrangements demonstrated high correlation with expected MAFs (Pearson correlation $r = 0.917$) (Figure 2F and Supplemental Table S5).

Copy Number Amplifications

Reference samples for CNAs consisted of cell line dilutions or clinical samples. Three reference cell lines were each diluted with paired normal DNA to simulate samples with varying ctDNA fractions (ranging from 20% to 100%), and amplifications with a reference copy number of eight or more were considered for evaluating performance of the assay. A total of 29 clinical cfDNA samples were confirmed to harbor an amplification by two orthogonal methods (FoundationOne NGS or ddPCR); ctDNA fraction was not assessable for all clinical samples (Supplemental Table S6).

Amplifications in genes with three or more or four or more targeted regions were evaluated (Supplemental Tables S1 and S6). The reference cell lines and clinical samples harbored a total of 43 expected amplifications in genes that had four or more targeted regions and 55 expected amplifications in genes that had three or more targeted regions (Supplemental Tables S1 and S6). For genes with four or more targeted regions (including *ERBB2* and *MET*), the assay achieved a sensitivity of 95% (95% CI, 82.9%–99.2%) and a PPV of 98% (95% CI, 85.9%–99.9%) (Table 1 and Supplemental Table S6). For genes with three or more targeted regions, the assay achieved a sensitivity of 89% (95% CI, 77.0%–95.4%) and a PPV of 98% (95% CI, 87.9%–99.8%) (Supplemental Table S6); the single FP was a *CCND1* amplification in a clinical cfDNA sample from a patient with breast cancer that was not detected by ddPCR.

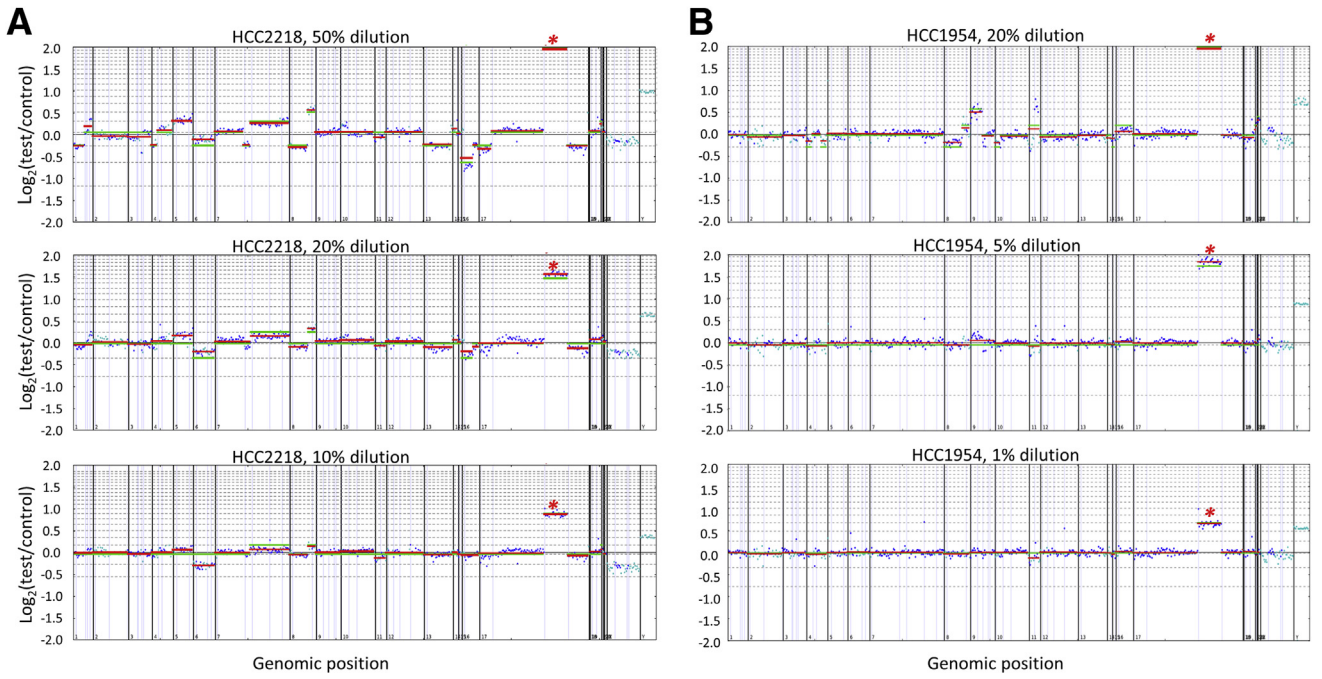


Figure 3 Assessment of *ERBB2* (*HER2*) amplification at lower tumor fraction. **A:** Example copy number amplification (CNA) data for the HCC2218 cell line (*ERBB2* copy number, 9) diluted with matched normal DNA to generate samples with different tumor fractions. *ERBB2* amplification detectable at 10% tumor fraction. **B:** Example CNA data for the HCC1954 cell line (*ERBB2* copy number, 17) diluted with matched normal DNA to generate samples with different tumor fractions. *ERBB2* amplification detectable at 1% tumor fraction. **A** and **B:** *y* Axes denote $\log_2$ ratio measurements of coverage obtained in test samples versus normal reference samples. Each point denotes a genomic region measured by the assay, and these are ordered by genomic position. **Red lines** indicate the average $\log_2$ ratio in a segment. **Asterisks** denote *ERBB2* amplification (chromosome 17).

The ability to call CNAs at lower tumor fractions was confirmed by further dilution of the two *ERBB2*-amplified cell lines HCC2218 (*ERBB2* copy number, 9; dilutions at 50%/20%/10%/5%/2.5%) and HCC1954 (*ERBB2* copy number, 17; dilutions at 100%/20%/16%/10%/5%/1%/0.5%); *ERBB2* amplification was detected at a tumor fraction of 10% for HCC2218 and at a tumor fraction of 1% for HCC1954 (Figure 3); no FP amplification calls were observed in the eight samples diluted to <20% tumor fraction. Therefore, amplifications can be detected at lower tumor fractions, but as expected, the tumor fraction required for CNA detection is highly dependent on the amplitude of the amplification (number of copies).

Evaluation of cfDNA Control Samples from Healthy Volunteers

The clinical specificity of the assay was further evaluated on 62 samples of cfDNA, taken from 24 different healthy individuals, that are not expected to contain somatic genomic alterations, as well as additional 38 duplicates from these samples. No FP alterations were identified, corresponding to zero FPs over 8.68 Mb of target sequence interrogated, which can be translated to a per-target-base specificity of >99.9999%.

Intrarun and Interrun Precision

The reproducibility of the assay was assessed from the library construction step through to sequence analysis. Precision was calculated by comparison in paired replicates of genomic alterations, including base substitutions, indels, and re-arrangements with an MAF ≥0.25% (Table 1 and Supplemental Table S8). To test intrarun precision, five clinical cfDNA samples were processed three times in parallel in the same run; a total of 12 genomic alterations were detected, and pairwise analysis of replicates resulted in intrarun precision of 100%. To test interrun precision, 13 samples were independently processed three times each; a total of 57 genomic alterations were detected, and pairwise analysis of replicates resulted in 100% interrun precision.

Assessment of FoundationACT on Clinical Samples

Concordance with Orthogonal Tests

To confirm performance of the assay in the clinical setting, results obtained by FoundationACT on clinical cfDNA samples were compared with orthogonal assays run on the same samples, including the following: i) FoundationOne NGS assay,[1] ii) ddPCR, iii) BEAMing, and iv) breakpoint PCR.

Concordance was evaluated across 115 clinical cfDNA samples, and a diversity of alterations were evaluated, including base substitutions, indels, re-arrangements, and CNA. The samples included 69 breast cancer cases [including 27 cfDNA samples from a phase 2 study in hormone receptor−positive, HER2-negative metastatic breast cancer (NCT01740336)],[24] 37 lung cancer cases, and

9 cases of other tumor types (Supplemental Table S9). Select genomic alterations were assessed, including alterations associated with sensitivity to existing therapeutics (eg, *EGFR* L858R, exon 19 deletion, *EML4-ALK* fusion, and *KIF5B-RET* fusion in lung cancer samples) and alterations associated with therapy resistance (eg, *EGFR* T790M, *ESR1* E380Q, Y537C/N/S, and D538G) (Supplemental Tables S10 and S11).

First, it was evaluated if genomic alterations detected by the FoundationACT ctDNA assay could be independently confirmed using the orthogonal assays. The 20 samples evaluated by FoundationOne NGS included 43 genomic alterations in 17 genes (35 base substitutions, 7 indels, and 1 re-arrangement) that were detected at MAF >5% (the limit of detection for FoundationOne) (Figure 4A and Supplemental Table S10). For 50 samples evaluated by ddPCR, concordance was assessed for 70 alterations in nine genes (63 base substitutions and 7 indels) (Figure 4B and Supplemental Table S10). For 13 hormone receptor−positive/HER2$^-$ metastatic breast cancer samples (from NCT01740336)[24] evaluated by BEAMing, concordance was assessed for 26 *ESR1* base substitutions (Figure 4C and Supplemental Table S10). The 15 samples evaluated by breakpoint PCR included a total of 15 rearrangements in six genes (Supplemental Table S10). Across the cfDNA samples and genomic alterations evaluated in the comparison of FoundationACT to orthogonal assays, all (128/128) alterations detected by the FoundationACT assay were also detected in the orthogonal assay, with no FPs (Figure 4 and Supplemental Table S10); there was high correlation between the MAF values observed by FoundationACT and orthogonal assays (Figure 4 and Supplemental Table S10).

Second, for the set of 27 hormone receptor−positive/HER2$^-$ metastatic breast cancer cfDNA samples (from NCT01740336),[24] it was evaluated whether 108 *ESR1* base substitution wild-type calls by FoundationACT could be confirmed by independent testing with BEAMing; 99.1% (107/108) of the *ESR1* calls were confirmed, with only one FN that was detected only by BEAMing (MAF, 0.38%) (Supplemental Table S11).

In addition, 29 clinical cfDNA samples were included as reference samples to evaluate analytical validity for CNAs; 35 reference amplifications in 13 genes were assessed (Supplemental Tables S6 and S9). In these samples, 91.4% (32/35) of reference amplifications identified by orthogonal methods could also detected by FoundationACT, and 97.0% (32/33) of the calls made were true positives.

Clinical Implementation of the FoundationACT Assay

On the basis of the analytical validation, FoundationACT has been deployed for routine clinical use to identify genomic alterations in ctDNA and guide patient care. The results of FoundationACT were evaluated on 884 consecutive routine clinical cases that were prospectively sequenced in our CLIA-certified/CAP-accredited/NY State−approved laboratory. cfDNA was sequenced to a median unique coverage depth of 8296×. Overall results from 860 of 884 (97.3%) of sequenced cfDNA samples passed quality control criteria and were reported out to clinicians and patients. Turnaround time from sample receipt to reporting of results was 12.3 ± 2.3 days (median ± SD).

The 860 reported cases were from 859 different patients with advanced cancer. The patient cohort included a diversity of tumor types, and the most common were NSCLC (n = 283), breast cancer (n = 128), cancer of unknown primary (n = 73), colorectal cancer (n = 67), and prostate cancer (n = 63) (Figure 5A). ctDNA, as evidenced by the presence of a somatic alteration (including reportable genomic alterations, variants of unknown significance, and synonymous mutations), was detected in 80.9% (696/860) of cfDNA samples, which is consistent with previous studies that evaluated the frequency of ctDNA detection in cfDNA samples from advanced solid cancers.[8]

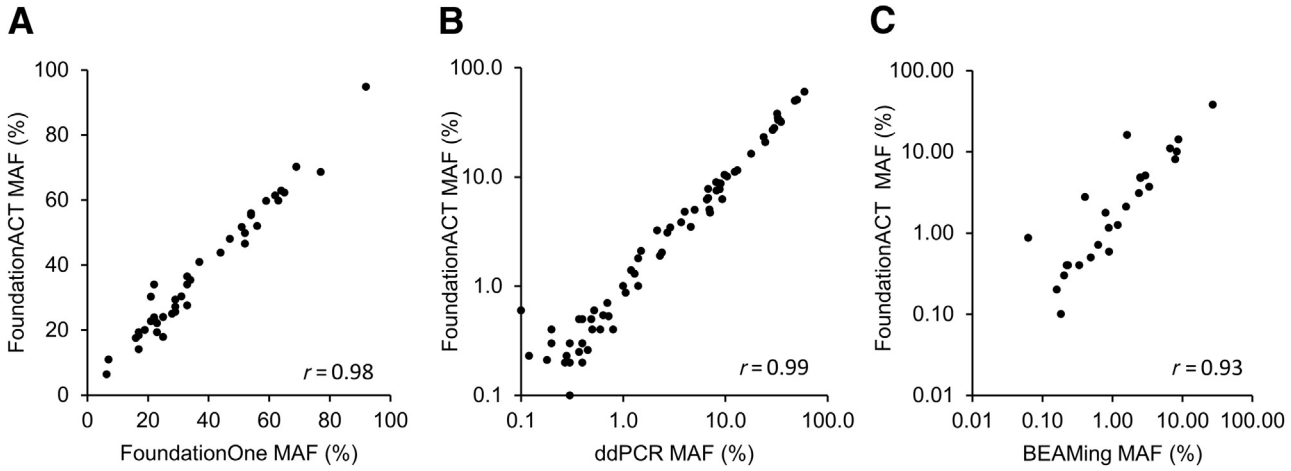


Figure 4 Validation of assay on clinical cfDNA samples by comparison with orthogonal assays performed on the same samples. Mutant allele frequencies (MAFs) observed by FoundationACT were correlated with MAFs observed using orthogonal assays, including FoundationOne next-generation sequencing (Pearson correlation r = 0.98; **A**), droplet digital PCR (ddPCR; Pearson correlation r = 0.99; **B**), and beads, emulsions, amplification, and magnetics (BEAMing; Pearson correlation r = 0.93; **C**).

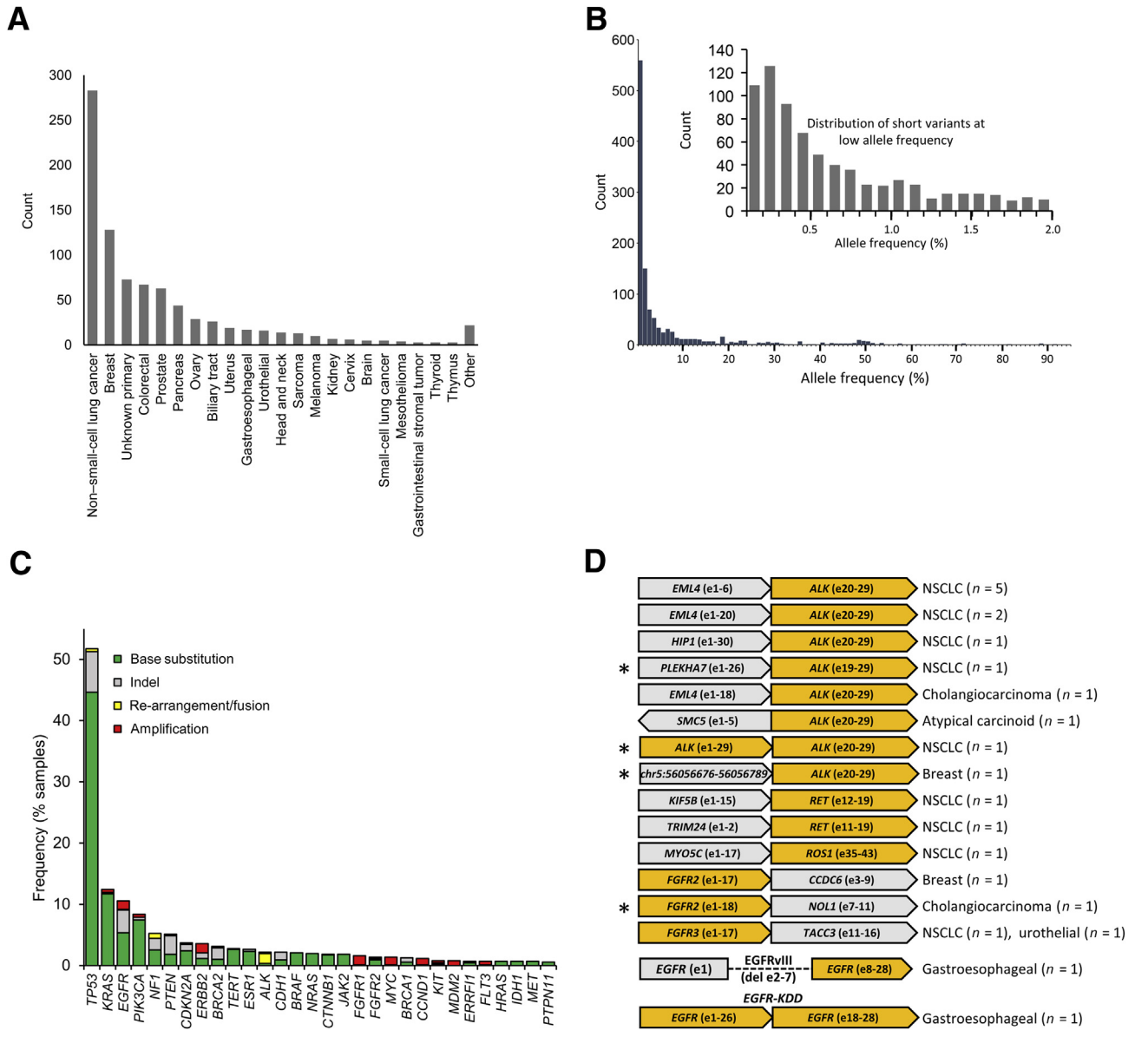
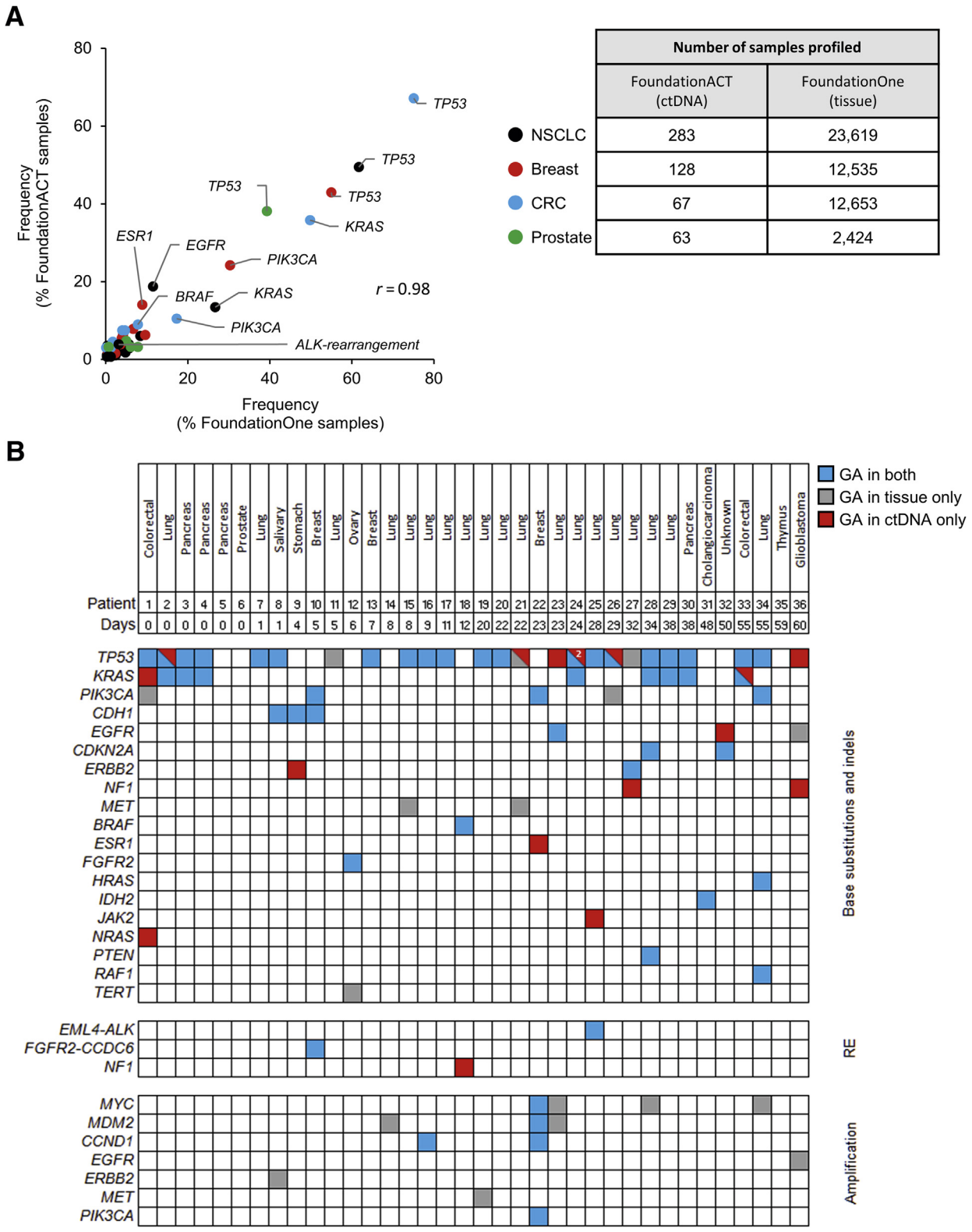


Figure 5 Clinical genomic profiling of ctDNA using the FoundationACT assay. **A:** Distribution of cancer types for the 860 cases that were successfully profiled. **B:** Histogram demonstrating the distribution of allele frequency for the 1252 reportable short variant mutations (variants of unknown significance not included) detected in the 860 cases. **Inset:** A detailed view for the subset of short variants at lower allele frequencies. **C:** Frequency of all reportable genomic alterations in most commonly altered genes among the 860 cases. Genes altered in five or more cases are shown. **D:** List of kinase fusions/re-arrangements detected. Arrows indicate the gene and specific exons involved in the fusion and directionality of the exons (e). **Yellow shading** indicates the portion of the re-arrangement that includes the intact kinase domain. **Asterisks** indicate a novel re-arrangement. EGFR KDD, epidermal growth factor receptor kinase domain duplication; Indel, insertion/deletion; NSCLC, non–small-cell lung cancer.

A total of 1400 reportable genomic alterations were detected, including 1021 base substitutions, 231 indels, 105 CNAs, and 43 gene fusions/re-arrangements; an additional 878 variants of unknown significance were also detected (Supplemental Table S12). At least one reportable genomic alteration (excluding variants of unknown significance) was observed in 70.6% of cases, with an average of 1.6 reportable genomic alterations per sample (range, 0 to 25). Reportable short variant mutations had a median MAF of 1.3%, with 31.6% (396/1252) of short variant mutations detected at low AFs (<0.5%) (Figure 5B).

Consistent with the tumor types represented in this series of cases, the most frequent reportable genomic alterations were observed in *TP53*, *KRAS*, *EGFR*, and *PIK3CA* (Figure 5C). Kinase re-arrangements were observed in 2.6% (22/869) of cases, including *ALK*, *ROS1*, *RET*, *FGFR2*, *FGFR3*, and *EGFR* re-arrangements (Figure 5D). As expected from prior genomic studies,[2,3] these kinase fusions were most frequently observed in NSCLC. Consistent with genomic profiling studies across diverse tumor types, kinase fusions were observed beyond the tumor types where they typically occur.[2,3,25,26] For example, *ALK* fusions were



Number of samples profiled		
	FoundationACT (ctDNA)	FoundationOne (tissue)
NSCLC	283	23,619
Breast	128	12,535
CRC	67	12,653
Prostate	63	2,424

Figure 6 Comparison of genomic profiling of ctDNA (FoundationACT) and tumor tissue samples (FoundationOne). **A:** The frequency of genomic alterations detected by FoundationACT was evaluated for non–small-cell lung cancer (NSCLC), breast cancer, colorectal cancer (CRC), and prostate cancer cases: for each cancer type, genes with at least two short variants or re-arrangements were included; short variants or re-arrangements were evaluated separately. The results were compared with those observed in our database of FoundationOne genomic profiling results from tissue biopsy specimens. **B:** Concordance between genomic alterations (GAs) detected in ctDNA and temporally matched tumor tissue from the same patient. Days between blood and tissue collection are shown. Concordant/shared genomic alterations are in blue, genomic alterations detected in tissue only are in gray, and genomic alterations detected in ctDNA only are in red. For samples with multiple unique mutations in a gene, the number of mutations is shown. Indel, insertion/deletion; RE, re-arrangement.

observed in tumor types other than NSCLC, including atypical carcinoid tumor, cholangiocarcinoma, and breast cancer. The activating *EGFR* re-arrangements *EGFRvIII* and EGFR kinase domain duplication (*EGFR-KDD*) that are associated with glioblastoma were each observed once in gastroesophageal junction adenocarcinoma (Figure 5D). Novel gene fusion partners were observed for *ALK* (*PLEKHA7-ALK*) and *FGFR2* (*FGFR2-NOL1*). *ALK* re-arrangements involving the kinase domain without a fusion partner were observed in two cases, including a duplication of the ALK kinase domain and an *ALK* intron 19 re-arrangement with intergenic space (Figure 5D). *ALK* variants that retain the kinase domain but lack the N-terminus and a fusion partner have been shown to be oncogenic and targetable; therefore, the re-arrangements observed herein are potentially clinically actionable.[27,28]

Two approaches were used to evaluate the results of the FoundationACT ctDNA assay in comparison to results from tissue samples that were assayed using a similar hybrid capture–based NGS assay (FoundationOne). First, frequencies of genomic alterations observed in samples sequenced using FoundationACT were compared versus our database of tissue samples sequenced using FoundationOne. Second, the concordance between genomic profiles of temporally matched blood and tissue samples that were collected from the same patient was evaluated.

For the most represented disease types in this series (NSCLC, breast, colorectal, and prostate cancers), the frequency of reportable short variant mutations and re-arrangements was evaluated. Overall, the observed frequencies from genomic profiling of ctDNA were highly consistent with frequencies observed in our data set of genomic profiling of tissue samples (Pearson correlation $r = 0.98$, $P < 0.0001$) (Figure 6A).

The FoundationACT data set is enriched in samples from patients who had received prior targeted therapy[29]; therefore, *EGFR* mutations in NSCLC and *ESR1* mutations in breast cancer were more frequent (>1.5-fold) in the FoundationACT data set compared with the tissue data set. Indeed, 28.3% (15/53) of NSCLC cases with an activating *EGFR* mutation also had a co-occurring *EGFR* T790M resistance mutation, including two cases with both *EGFR* T790M and a concurrent C797S osimertinib resistance mutation. Other genomic alterations associated with resistance were observed, including *ALK* mutation in *ALK* fusion-positive NSCLC cases, *BRCA2* reversion mutations in breast cancer, and *EGFR* extracellular domain mutations in colorectal cancer.

For 36 patients in this series, genomic profiling results were available for temporally matched blood and tissue samples collected from the same patient within 60 days of each other (median, 16 days; range, 0 to 60 days); only reportable genomic alterations covered by both assays were assessed for concordance (Figure 6B). Matched samples were from patients with NSCLC ($n = 19$), pancreatic cancer ($n = 4$), breast cancer ($n = 3$), colorectal cancer ($n = 2$), and other tumor types ($n = 8$). Of the 36 cases, 3 had no evaluable genomic alterations in tissue and, similarly, no genomic alterations were detected in the matched blood samples. For the genes covered by both assays, a total of 68 reportable genomic alterations (53 short variants mutations, 13 CNAs, and 2 re-arrangements) were detected in 33 tissue samples, of which 75.0% (51/68) were also detected in temporally matched cfDNA, including 83.0% (44/53) of short variant mutations, 38.5% (5/13) of CNAs, and 100% (2/2) re-arrangements. Conversely, 75.0% (51/68) of genomic alterations detected in cfDNA were also detected in tissue. Overall, of the 33 cases with a genomic alteration in tissue, 87.9% (29/33) had at least one concordant genomic alteration in cfDNA.

Discussion

Prospective genomic profiling of tumor tissue samples has been adopted in routine clinical practice to guide genomic biomarker–based selection of approved targeted therapies or to facilitate enrollment onto genomically matched clinical trials.[2,3] However, for those cases where acquisition of a contemporaneous tissue sample is not feasible, ctDNA released into the blood by tumors provides patients with an opportunity to noninvasively evaluate tumor-derived genomic alterations. The current paradigm for tissue- versus blood-based testing is highlighted by biomarker testing for EGFR TKIs: for patients who progress taking first- or second-generation EGFR TKIs, the National Comprehensive Cancer Network recommends *EGFR* T790M biomarker testing using cfDNA-based testing if tissue biopsy is not feasible, and conversely recommends reflex to tissue-based testing if an upfront cfDNA test is negative for T790M; therefore, tissue and cfDNA-based testing can be complementary approaches for the detection of genomic biomarkers.

Herein, we developed and performed an analytical validation of a hybrid capture–based NGS assay (FoundationACT) to enable clinical genomic profiling of ctDNA from blood of patients with cancer to detect base substitutions, indels, re-arrangements, and CNAs in a panel of 62 cancer-related genes. Our broad-based testing approach enables unbiased detection of multiple cancer drivers without prior assumptions about the prevalence of the genomic alterations present in any given cancer type, and in contrast to sequential testing of single biomarkers, it may allow efficient detection of both frequent genomic alterations and rare but clinically important targetable drivers.

A key consideration in our assay development was to sensitively detect genomic alterations in ctDNA at the low allele frequencies that are often observed in clinical samples (Figure 5B)[8,11,15,30] while minimizing FPs. This was achieved by sequencing to sufficiently high coverage depth to obtain multiple observations for most DNA fragments in each sample and using fragment barcodes to accurately

detect and exclude errors introduced during library preparation and sequencing.

The analytical validation was performed on 2666 test alterations from reference samples derived from cell line models, synthetic gene fusions, and clinical samples. The overall performance of the assay was high for short variants (AF $\geq$ 0.5%) and re-arrangements (AF $\geq$ 0.25%) that were detected with a sensitivity of >99% [95% CI, 99.1%−99.4% (short variants) and 56.1%−100% (re-arrangements)] (Supplemental Tables S3 and S5); sensitivity remained high at lower MAFs, and high PPV of >99% was achieved across the range of evaluated MAFs down to MAF 0.125%. The MAFs reported by the assay significantly correlated to expected MAFs.

On the basis of analysis of reference samples that included cell line dilutions and clinical cfDNA samples, it was established that CNA calling thresholds had high sensitivity (>95%; 95% CI, 82.9%−99.2%) to detect amplifications at copy number eight or greater and in samples with ≥20% ctDNA fraction. Overall, CNA calling demonstrated high PPV (>97%; 95% CI, 85.9%−99.9%). CNA calling thresholds are an integrated measure of both tumor fraction and magnitude of amplification; therefore, higher-level amplifications are detectable at lower tumor fractions (Figure 3). Indeed, evaluation of *ERBB2*-amplified cell line dilutions illustrates the ability to detect high-level amplifications at low tumor fractions.

The performance of FoundationACT was further validated on 115 clinical cfDNA samples, and genomic alterations detected by the assay were highly concordant with multiple orthogonal approaches run on the same cfDNA samples, including the FoundationOne NGS assay, ddPCR, BEAMing, and breakpoint PCR.

FoundationACT has been clinically implemented in our CLIA-certified/CAP-accredited/NY State−approved laboratory to prospectively identify genomic alterations in ctDNA that are reported to patients and physicians to guide routine clinical care. The results of prospective genomic profiling from 860 blood samples were assessed from patients with diverse cancer types that were sequenced using the FoundationACT assay. Mutations at low MAF were frequently detected, demonstrating the importance of achieving high sensitivity and specificity at low AF.

Diverse mutations that drive acquired resistance to targeted therapies were detected, including *ALK* mutations (ALK TKI resistance), *BRCA2* reversion mutations [poly (ADP-ribose) polymerase inhibitor resistance], *EGFR* T790M (first- and second-generation EGFR TKI resistance), *EGFR* C797S (third-generation EGFR TKI resistance), *EGFR* extracellular domain mutations (anti-EGFR antibody resistance), and *ESR1* ligand-binding domain mutations (aromatase inhibitor resistance). Noninvasive blood-based genomic profiling may be particularly appropriate for longitudinal testing that is required to identify novel acquired genomic alterations associated with resistance to targeted therapies, and detection of such resistance alterations in ctDNA at progression on initial targeted therapy could guide the selection of subsequent lines of targeted therapies. Broad gene panels are advantageous for capturing the diversity of potential genomic alterations that can drive resistance to targeted therapies[31−33]; however, as we begin to understand the full scope of potential acquired genomic alterations that drive resistance, development of more focused panels may allow a cost-effective way to serially monitor resistance and disease burden over the course of therapeutic treatment.

Genomic profiling studies of tissue have demonstrated that kinase fusions can be found at low frequencies in a broad spectrum of tumor types, and increasingly, responses to targeted therapies have been documented in patients with kinase fusions in new tumor types, where they have not been previously observed.[27,34,35] In this study, diverse kinase fusions, including in tumor types where they are not commonly found, were detected, demonstrating the importance of broad-based sequencing to capture uncommon cancer drivers. For example, a patient with an atypical carcinoid tumor had previously responded to treatment with the ALK inhibitor alectinib on the basis of a novel *SMC5-ALK* re-arrangement detected using FoundationACT[27]; a second sample from the same patient was sequenced in the series of cases presented in this study, and a newly arising mutation (*ALK* I1171T) that has been associated with resistance to alectinib was identified.[36−38] This case demonstrates the clinical utility of genomic profiling of ctDNA for detecting novel actionable kinase fusions in unexpected tumor types and the ability of serial testing to identify resistance alterations that may inform subsequent treatment selection.

To demonstrate that FoundationACT identifies the expected genomic alterations in routine clinical samples, tissue-based testing was used as a reference standard, and it was asked whether genomic profiling of ctDNA could closely recapitulate the results obtained by tissue-based testing. First, in each of the most frequent tumor types evaluated, the FoundationACT assay detected genomic alterations at frequencies that were consistent with the expected frequencies based on our database of sequenced tissue biopsy specimens. Second, a concordance analysis on genomic profiling of temporally matched samples demonstrated that the ctDNA assay could sensitively detect genomic alterations that were confirmed to be present in paired tissue biopsy specimens: 75% of all genomic alterations and 83% of short variant mutations that were detected in tissue were also detected in cfDNA.

Additional genomic alterations were detected only in blood samples but not in paired tissue samples, including *TP53* mutations in NSCLC and *KRAS* and *NRAS* mutations in colorectal cancer; a *JAK2* V617F was also detected in an NSCLC cfDNA sample and is likely associated with clonal hematopoiesis and not a bona fide driver of NSCLC.[39−41] The presence of additional genomic alterations may be associated with clonal evolution in response to therapy and release of ctDNA into the blood from more than one metastatic site; future studies comparing genomic profiles in cfDNA samples with multiregional tissue biopsy specimens of different

metastatic sites will further inform our understanding of such mutations. Indeed, other studies have described subclonal *TP53* mutations[42] and *TP53* alterations that are unique to cfDNA in NSCLC[41,43]; *RAS* mutations that emerge as a mechanism of acquired resistance to anti-EGFR antibodies were more frequently detected in cfDNA compared with tissue in one study of colorectal cancer.[44] The clinical implications of alterations that are detected in cfDNA, but not in tissue biopsy specimens, are currently unclear and would benefit from clinical trials comparing the performance of genomic biomarkers detected in cfDNA versus tissue.

The clinical series presented herein included a broad range of cancer types. Further tumor type−specific studies are warranted to understand the performance of genomic profiling of ctDNA in the context of each cancer type. For example, a recent study demonstrated robust performance of the FoundationACT assay for estrogen receptor−positive breast cancer[29]; blood-based genomic profiling may be challenging for disease types, such as glioma, that often do not release sufficient ctDNA into the blood.[8,45] Additional studies of clinical validity and clinical utility are required for ctDNA assays,[46] and the comparison of ctDNA assays to approved tissue-based predictive biomarker tests will inform the relative roles of ctDNA versus tissue-based genomic profiling in the management of patients with cancer.

In summary, the FoundationACT assay underwent rigorous analytical validation testing on 2666 reference alterations that capture the diversity of genes and alteration types targeted by the assay across the range of allele frequencies assessed by the assay. The ability of the assay to identify base substitutions, indels, re-arrangements, and CNAs was validated with high levels of sensitivity, specificity, and reproducibility. In routine genomic profiling of clinical samples, FoundationACT detected genomic alterations at frequencies comparable to those observed in tissue-based genomic profiling; in a subset of clinical samples with genomic profiling results from temporally matched blood and tissue samples, FoundationACT was determined to be sensitive for the detection of genomic alterations that were identified in tissue. The development of this blood-based ctDNA assay may provide an alternative or complementary approach to tissue-based genomic testing for patients with cancer.

Supplemental Data

Supplemental material for this article can be found at *https://doi.org/10.1016/j.jmoldx.2018.05.004*.

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