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Comprehensive genome profiling by next generation sequencing of circulating tumor DNA in solid tumors: a single academic institution experience

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Abstract

Background: Recently, new evidence of the next-generation sequencing (NGS) liquid biopsy utility in clinical practice has been developed. This assay is emerging as a new promising tool to use as a noninvasive biomarker for cancer mutation profiling. Additional data supporting the clinical validity of cell free DNA (cfDNA) based testing is necessary to inform optimal use of these assays in the clinic.

Materials and methods: A total of 398 cancer patients were analyzed by FoundationOne Liquid Analysis (F1LA), a genomic profiling assay and by standard NGS diagnostic ThermoFisher platform. The association between diagnostic technique was evaluated using a Poisson regression model. FoundationOne Liquid (F1L) and FoundationOne Liquid CDx (F1LCDx) detect 70 and 324 cancer-related genes alterations, respectively, including genomic signatures tumor fraction, blood tumor mutational burden (only for the 324 genes version), and microsatellite instability high status. Both assays used a single DNA extraction method to obtain cfDNA. The real-life clinical impact and feasibility of F1L and F1LCDx were evaluated across different solid tumors in our department.

Results: Between 1 January 2019 and 28 February 2021, 398 samples of different tumor types from 398 patients were analyzed (overall success rate: 92%, in FoundationOne Liquid CDx Analysis success rate: 97%). Most frequent molecular alterations were *TP53* (74), *APC* (40), *DNMT3A* (39), *KRAS* (23). The comprehensive clinical impact of F1LA compared with standard diagnostic was 64.7% *versus* 22.1% [risk ratio (RR) $= 2.94$; $p < 0.001$] and the potential clinical impact was 58.6% *versus* 11.0% (RR $= 5.32$; $p < 0.001$), respectively. Furthermore, some clinical cases were selected, in which F1LA detected actionable alterations offering an unexpected therapeutic choice.

Conclusions: Although additional studies are needed to better select patients and setting, NGS F1LA is a useful, noninvasive, and repeatable assay to guide therapeutic choice in oncology. It provides a snapshot of cancer heterogeneity profile that could be incorporated in routinely clinical practice.

Keywords: cfDNA, clinical trials, liquid biopsy, mCRC, precision medicine

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Introduction

In the precision medicine era, cancer treatment optimizes the clinical benefits for each patient by choosing targeted treatments based on that tumor's unique genetic profile, avoiding ineffective therapies. However, personalized treatment requires comprehensive and precise genetic profiling of the patient's tumor.[1,2]

During the last years, several comprehensive genomic profiling (CGP) assays have been developed and integrated in clinical practice, allowing identification of target therapy, monitoring therapeutic response, treatment resistance, and disease relapse.[3] Among these, the next-generation sequencing (NGS) assay has offered unprecedented progress in uncovering cancer genome characteristics and facilitating personalized cancer therapy due to its outstanding accuracy, sensitivity, and high throughput.[4] NGS-based tumor CGP detects different classes of somatic genomic alteration such as base pair substitutions, copy number variations, insertions/deletions, and rearrangements. This approach is increasingly being utilized to optimize patients' treatments based on several oncogenic drivers.[5]

In the tailored medicine, limitations of tissue biopsy include tumor tissue accessibility, invasive procedures and turnaround times, and lack of representation of whole tumor heterogeneity. Recently, liquid biopsy emerged as a noninvasive alternative approach able to overcome these limitations. Liquid biopsy is a minimally invasive test to evaluate circulating cell-free DNA (cfDNA) from the peripheral blood of cancer patients, using CGP, based on digital polymerase chain reaction and NGS technology.[6]

In the last decade, many NGS panel tests for liquid assay have been developed, with improvement in sensitivity, specificity, accuracy, feasibility, accessible costs, and real-world evidence.

Therefore, liquid biopsy is moving toward to become mainstream in precision medicine to profile the real time heterogeneity of the patients' cancer, with an affordable cost and in less time.[7–9]

FoundationOne Liquid (F1L) assay was a target-specific NGS-based device for liquid biopsy of FoundationMedicine, that utilized cfDNA isolated from plasma derived from anticoagulated peripheral whole blood and used targeted high throughput hybridization-based capture technology to detect and report in a 70 targeted genes panel substitutions, insertions and deletions (indels), copy number alterations (CNAs), selected gene rearrangements in 7 genes, and genomic signature microsatellite instability high (MSI-H) status.[10]

From September 2020, the new released version of FoundationOne Liquid CDx (F1LCDx) replaced the previous one. F1LCDx is a CGP test that analyses more than 300 cancer-related genes and multiple genomic signatures. In particular, it detects in 324 targeted genes panel (309 genes with complete exonic coverage and 15 genes with select noncoding coverage) molecular alterations: substitutions and indels in 311 genes, CNAs in 3 genes, selected genomic rearrangements in 4 genes, and genomic signatures including tumor fraction (TF), blood tumor mutational burden (bTMB), and MSI-H status.[11–13]

Between October and November 2020, the Food and Drug Administration (FDA) approved F1LCDx as a companion diagnostic device for multiple biomarkers detected in cfDNA (ALK rearrangement, EGFR exon 19 deletion and EGFR exon 21 L858R substitution in lung cancer, BRCA1, BRCA2, ATM alterations in prostate cancer, BRCA1 and BRCA2 in ovarian cancer, PIK3CA mutations in breast cancer).[14]

In this work, we investigated clinical impact of FoundationOne Liquid Analysis (F1LA) on peripheral blood cfDNA from cancer patients treated in our institution using both F1L (January 2019–August 2020) and F1LCDx (September 2020–February 2021). We investigated molecular alterations in different tumor types: colorectal cancer (CRC), non-small cell lung cancer (NSCLC), gastric cancer (GC), pancreatic cancer (PC), biliary tract cancer (BTC), breast cancer (BC), and other cancer (including all other cancer types).

Our goal was to determine the real-world impact of routine incorporation of FoundationOne Liquid Analysis across different cancer types, with a focus on CRC.

Materials and methods

Characteristics of the population

Between 1 January 2019 and 28 February 2021, 398 patients (⩾18 years) were candidates for F1LA according to the oncologist's choice, based on clinical patient's needs.

The institution has a Multidisciplinary Molecular Tumor Board that was established in June 2019, where the cases were referred and reviewed from this date. Before June 2019, the reference cases were evaluated by the Multidisciplinary Oncology Groups (MOG) of different tumor types.

Patients provided written informed consent for an institutional review board–approved protocol for collection of plasma and tumor DNA profiling and related clinical data within the I-Cure research program. All data were collected and stored in an established genomic and clinical database and analyzed to determine the real-world impact of F1LA.

Before or after surgery, before oncological therapy or at least 2 weeks after the previous treatment, two anti-coagulated peripheral whole blood tubes (8.5 ml per tube provided by FoundationMedicine) were collected.[12] Immediately after blood collection, the blood tubes were sent at room temperature[12] to FoundationMedicine and arrived at the laboratory within 3 days.

Standard NGS diagnostic analysis

In addition, based on clinical practice guidelines, patients were assessed for mutations, according to the cancer subtype, with an NGS ThermoFisher platform. The ION Torrent Personal Genome Machine (PGM) technology allows the massive parallel sequencing of DNA libraries, of several different samples, using an approach based on the PH variations that occur at the moment of incorporation of the single deoxyribonucleotide into the reaction catalyzed by the DNA polymerase. Around 10 ng of DNA was used to prepare the sequencing libraries. The libraries were prepared with the IonAmpliSeq™ Library kit 2.0 (Thermo Fisher Scientific, Waltham, MA, USA) and with 2 types of primer pool: IonAmpliSeq Colon and Lung Cancer Research Panel v2, that analyze 504 mutational hotspots and targeted regions in 22 genes, and AmpliSeq Cancer Hotspot Panel v2 that analyze 2800 mutational hotspots and targeted regions in 50 genes.

Liquid biopsy analysis

F1L and F1LCDx assay analysis were performed from January 2019 to August 2020 and from September 2020 to February 2021, respectively.

Both assays used a single DNA extraction method to obtain cfDNA. The test requires about $\geqslant$ 25 ng cfDNA. Extracted cfDNA undergoes to whole-genome library construction. The libraries are sequenced with deep coverage using the NovaSeq® 6000 platform. Sequence data are processed using a custom analysis pipeline designed to detect genomic alterations, substitutions and indels, CNAs, genomic rearrangements, TF, bTMB and MSI-H status.[12,13]

For F1LCDx the targeted high throughput hybridization-based capture selected libraries identify 324 cancer-related genes: 309 genes with complete exonic (coding) coverage and 15 genes with only select noncoding coverage. The test also detects the genomic signatures bTMB (expressed in number of mutations/Mb unit), MSI-H status, and TF for copy number alterations (expressed in percentage). Therefore, variant allele frequency (VAF) for short variants and rearrangements is also calculated and reported in percentage.[11–13]

On the other hand, F1L interrogated 70 genes, 35 genes with entire coding sequence and 35 genes with selected exonic coverage. Among these, 7 genes also have selected intronic coverages for rearrangement detection.[10–15]

The MSI status is determined analyzing 2000 repetitive loci to identify what repeat lengths are present in the sample. A locus is considered unstable when it presents a repeat length of an internal database. The fraction of unstable loci is calculated and only the loci with adequate coverage for consideration for the sample are considered. Samples with $>0.5\%$ unstable loci are considered MSI-High. The MSI algorithm is based on genome wide analysis of 1765 microsatellite loci.[11–13]

Blood tumor mutational burden (bTMB) is measured by the number of all synonymous and nonsynonymous variants divided per area of the coding genome region, and after the removal of known and likely oncogenic driver events and potential germline variants (according to Single Nucleotide Polymorphism database and Exome Aggregation Consortium). It corresponds to the number of total variants counted or approximately 750 kilobases (kb). The resulting number is reported in units of mutations per megabase (mut/Mb). bTMB is calculated based on variants with an allele frequency of $\geqslant 0.5\%$.[11–13]

Tumor fraction (TF) is the percentage of circulating tumor DNA (ctDNA) present in a cell-free

DNA (cfDNA) sample. It is computationally derived from the observed level of aneuploidy in the sample and is considered elevated when ctDNA levels are high enough that aneuploidy can be detected.[11–13]

Approved results are saved by automated software, relevant information is merged with demographic, clinical and therapeutic (standard and experimental therapy) information, then a completed F1LCDx report is generated.[11–13] Validated genes, biomarkers, clinical trials list, and proposed therapies are periodically updated with the new knowledge about cancer molecular profiles and cancer therapies.[11,12,15]

Analysis failures include suboptimal cfDNA amount for testing process, inadequate cfDNA quality to pass quality controls, or analysis failure for a laboratory error.

Clinical analysis

Molecular and clinical information was matched with F1LA data for each patient to evaluate the clinical impact of F1LA in clinical practice.

The comprehensive clinical impact (CCI) of F1LA or using standard diagnostic was defined as the identification of molecular alterations useful for the patient's clinical management. Standard diagnostic was the routine NGS molecular analysis in our local anatomopathological laboratory. Although it was done on archival tumor tissue obtained at time of diagnosis, in clinical practice it is sometimes the only sample available due to the impossibility to repeat the biopsy (both for cancer characteristics and for patient choice) during the cancer history. Therefore, standard diagnostic clinical utility was compared with F1LA sampling at each time point.

Moreover, the potential clinical impact (PCI) of both diagnostic techniques was defined as the identification of at least one clinical trial match based on molecular profile.

The association between diagnostic technique and both CCI and PCI was evaluated using a Poisson regression model adjusted for age, gender, Eastern Cooperative Oncology Group (ECOG), and diagnosis. Results were expressed as risk ratios (RRs) and 95% confidence intervals (CIs).

Results

Population analysis

Between 1 January 2019 and 28 February 2021, 398 patients were candidate to perform a liquid biopsy. The patients were divided in seven different subgroups according to different solid tumor types. Characteristics of the patient population are shown in Table 1. Median age (60.86 years; range: 20–86 years) and mean age (59.98 years) were similar. All patients were Caucasian, 43.5% were female, and 56.5% were male. The Performance Status according to ECOG was 0 or 1 in 93% of patients.

For 119 patients out of 398 (29.9%) tissue molecular testing was not possible or was incomplete due to insufficient tissue or difficult biopsy: 17 patients (4.3%) were diagnosed with CRC; 40 (10%) with NSCLC; 10 (2.5%) with GC; 12 (3%) with PC; 4 (1%) with BTC; 3 (0.8%) with BC; 33 (8.3%) with other cancer (including all other cancer types).

Only 8.3% of F1LA were performed before (after diagnosis) or after radical surgery (then follow-up or adjuvant therapy or prosecution of chemotherapy); majority of F1LA (41%) was performed at baseline of first line; all others were performed at subsequent lines.

From 1 January 2019 to 31 August 2020, 255 F1L were performed; between 1 September 2020 and 28 February 2021, 143 F1LCDx were performed. A total of 398 F1LA were divided in seven subgroups according to the tumor samples types (Figure 1(a)–(c)). In both F1L and F1LCDx analysis, the most frequent tumor subtypes were CRC, NSCLC, GC, and other cancers (this subtype included all other tumor types).

Overall, 367 samples out of 398 completed the analysis (success rate: 92%). Among the 31 samples (8%) that failed the analysis, in 20 samples (5%) there was not enough cfDNA (TIFA–Tumoral-DNA Insufficient For the Analysis), while in 11 samples (3%) the Foundation Medicine laboratory was not able to complete the analysis (FMI Lab Fail) (Supplementary Figure 1A–D).

Moreover, the failed analysis decreased when it was switched from F1L (failure rate: 11%) to F1LCDx (failure rate: 3%). In fact, 228 samples out of 255 (success rate: 89%) completed analysis with F1L while 139 samples out of 143 (success

Table 1. Characteristics of the patients' population.

	Total *N* (%)	CRC	NSCLC	GC	PC	BTC	BC	Others
Age								
Median	60.86 (20–86)	61.45 (30–81)	63.69 (35–82)	55.99 (27–86)	63.48 (35–79)	66.51 (45–74)	53.56 (33–67)	57.78 (20–86)
Mean	59.98	61.27	62.79	57.27	62.63	63.69	53.07	56.58
Gender								
Female	173 (43.5%)	48 (12%)	31 (7.8%)	17 (4.3%)	7 (1.8%)	13 (3.3%)	29 (7.3%)	28 (7%)
Male	225 (56.5%)	66 (16.6%)	61 (15.3%)	41 (10.3%)	17 (4.3%)	10 (2.5%)	0 (0%)	30 (7.5%)
Race								
Caucasian	398 (100%)	114 (28%)	92 (23%)	58 (15%)	24 (6%)	23 (6%)	29 (7%)	58 (15%)
ECOG PS								
0	199 (50%)	55 (13.8%)	39 (9.8%)	33 (8.3%)	9 (2.3%)	15 (3.8%)	21 (5.3%)	27 (6.7%)
1	171 (43%)	48 (12%)	44 (11%)	21 (5.7%)	14 (3.3%)	8 (2%)	8 (2%)	28 (7%)
2	23 (5.8%)	7 (1.7%)	9 (2.3%)	3 (0.8%)	1 (0.2%)	0 (0%)	0 (0%)	3 (0.8%)
3	5 (1.3%)	4 (1.1%)	0 (0%)	1 (0.2%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Systemic anti-cancer therapies at the time of the test								
Before/after radical surgery/ adjuvant	33 (8.3%)	5 (1.3%)	3 (0.8%)	9 (2.3%)	2 (0.5%)	6 (1.5%)	1 (0.3%)	7 (1.8%)
First line	163 (41.0%)	31 (7.8%)	50 (12.6%)	26 (6.5%)	11 (2.8%)	10 (2.5%)	10 (2.5%)	25 (6.3%)
Second line	83 (20.9%)	21 (5.3%)	23 (5.8%)	14 (3.5%)	7 (1.8%)	6 (1.5%)	2 (0.5%)	10 (2.5%)
Advanced lines	119 (29.9%)	57 (14.3%)	16 (4%)	9 (2.3%)	4 (1%)	1 (0.3%)	16 (4%)	16 (4%)

BC, breast cancer; BTC, biliary tract cancer; CRC, colorectal cancer; ECOG PS, Eastern Cooperative Oncology Group Performance Status; GC, gastric cancer; NSCLC, non-small cell lung cancer; PC, pancreatic cancer.

rate: 97%) completed analysis with F1LCDx (Supplementary Figure 1A–D).

Regarding failure reasons, among 27 F1L failed (failure rate: 11%), 16 samples failed (failure rate: 7%) for suboptimal cfDNA amount (TIFA), and 11 samples (failure rate: 4%) failed for cfDNA inadequate quality or a laboratory error (FMI LAB FAIL). On the contrary, the only 4 F1LCDx (failure rate: 3%) failed were for TIFA (Supplementary Figure 1A–D).

Molecular analysis

The molecular analysis was performed on F1LCDx samples in order to have a better genetic characterization in larger panel of molecular alterations. A total of 139 F1LCDx completed analysis were included, while 4 failed analysis were excluded. The genetic alterations are shown in Figure 2(a)–(c). All molecular alterations founded in 139 reports divided for gene were reported. The molecular alterations were also classified for type (substitutions/indels, gene fusions, gene truncations, rearrangements, gene deletions, amplifications). All substitutions/indels with VAF < 0.5 were not counted. In the Figure 2(c), a detail of the most frequent alterations is depicted: TP53 (74), APC (40), DNMT3A (39), KRAS (23). MSI status, bTMB and TF were also analyzed in the different tumor types (Supplementary Table 1). All samples had MSS status; 11 samples had bTMB-high ($\geqslant$17 mutations), 2 samples had bTMB undetermined; 90 samples had TF undetermined.

Furthermore, the percentage of samples with specific pathway alterations was evaluated according to the cancer subtype (Table 2).

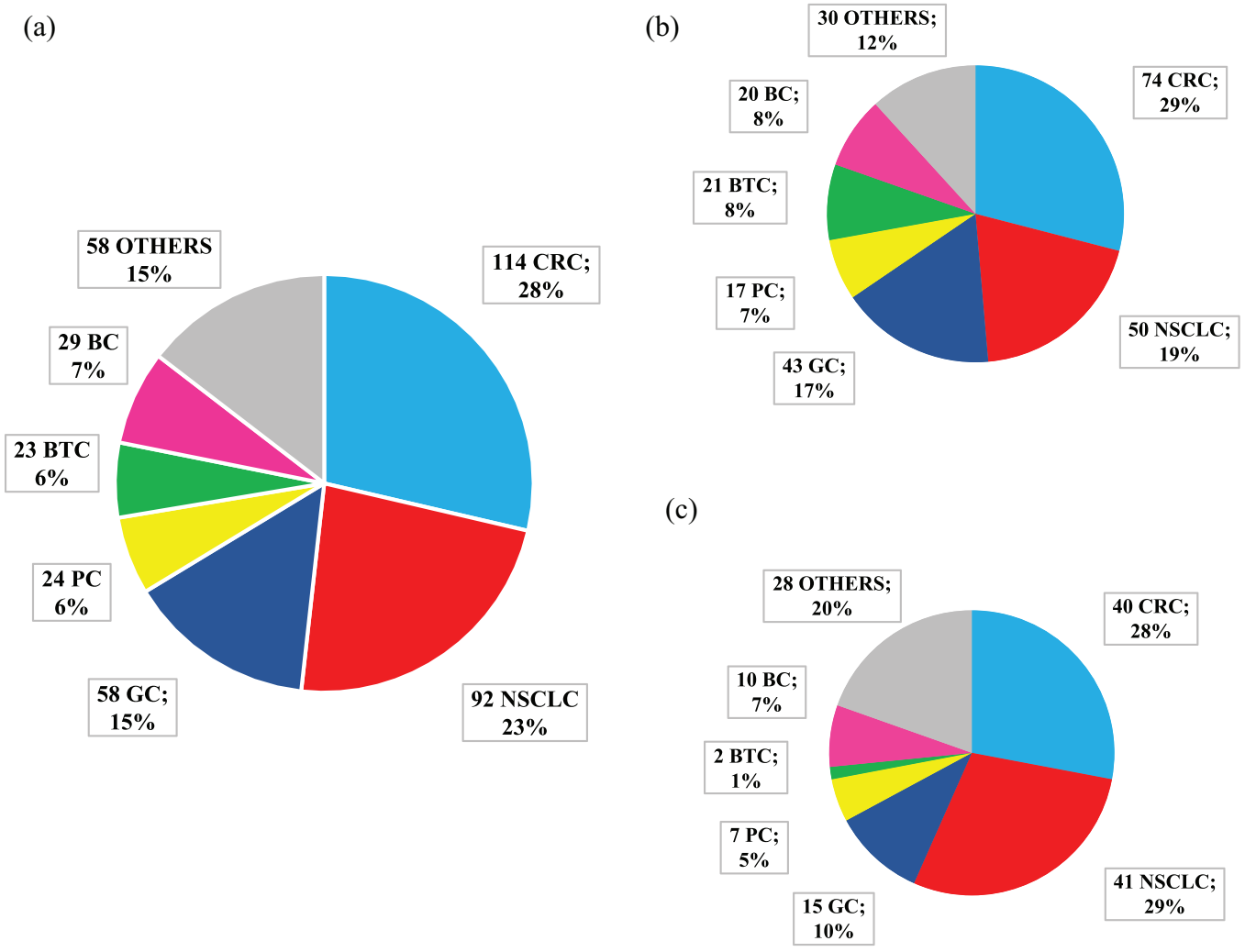

Figure 1. Tumor samples types. (a) Total tumor samples types: 398 liquid samples, both for Foundation Liquid analysis (70) and for Foundation Liquid CDx analysis (324): number of samples for type of tumors and percentage of samples for type of tumor. (b) Tumor samples types – liquid analysis (70): 255 liquid samples for Foundation Liquid analysis (70): number of samples for type of tumors and percentage of samples for type of tumor. (c) Tumor samples types – liquid CDx analysis (324): 143 liquid samples for Foundation Liquid CDx analysis (324): number of samples for type of tumors and percentage of samples for type of tumor.
CRC, colorectal cancer.

All cancer subtypes samples had chromatin remodeling and TP53 pathways altered. CRC had the greatest number of altered pathways with alterations in RAS, WNT/APC, HRR, PI3 K/PTEN/AKT/mTOR, chromatin remodeling, and TP53 pathways (Table 2). In BTC, chromatin remodeling, TP53, and other pathways were the only altered ones. In GC, the most frequent altered pathways were TP53, PI3 K/PTEN/AKT/mTOR, chromatin remodeling, and others; in PC the most frequent altered one was RAS; in BC were hormone receptor and PI3 K/PTEN/AKT/mTOR (Table 2).

Clinical analysis

The applications of F1LA in clinical practice were evaluated. The clinical impact of F1LA or standard diagnostic analysis is shown in Supplementary Table 2A–B.

F1LA identified molecular alterations not detected by standard diagnostic on 189 samples (47.4%) *versus* 18 samples (4.5%) on which only standard diagnostic had a clinical impact (Supplementary Table 2A). CCI with F1LA was 64.9% compared with 22.1% with standard diagnostic (RR = 2.94, 95% CI: 2.43–3.57, $p < 0.001$).

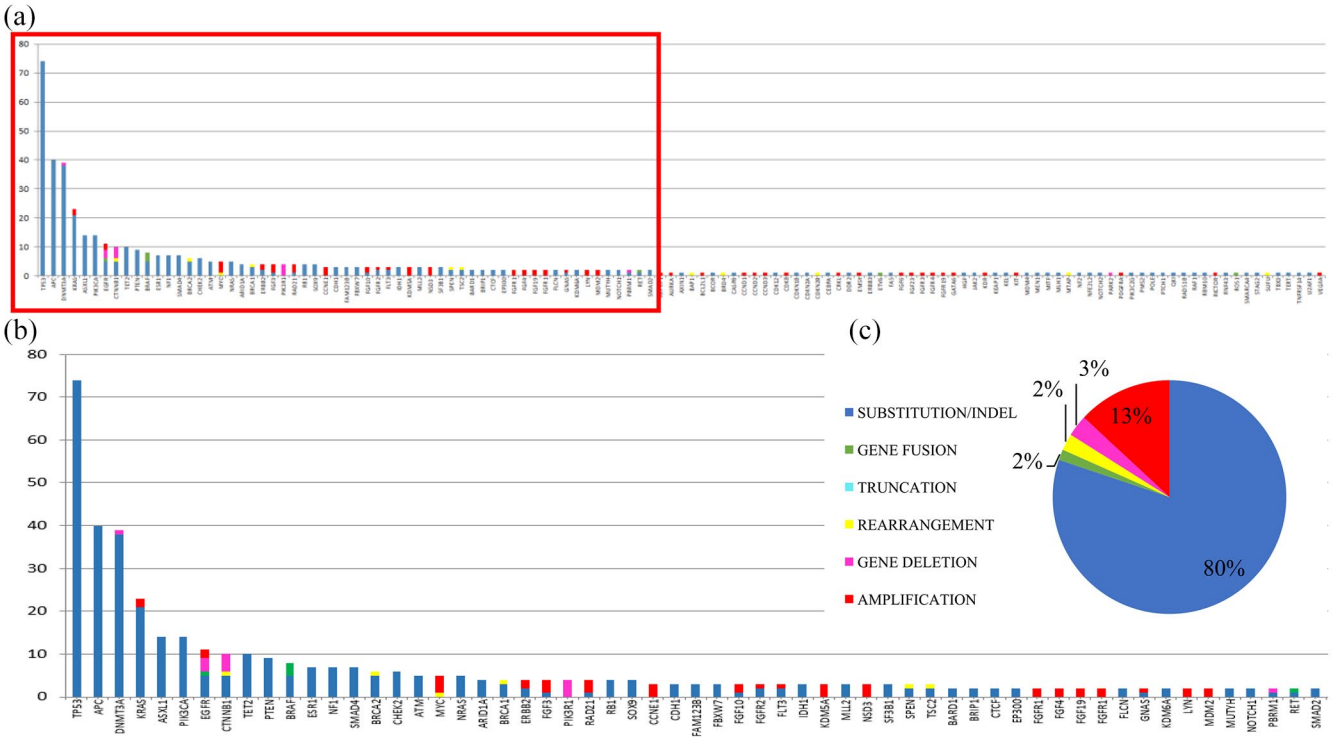

Figure 2. Genetic alterations in the population of Liquid CDx samples. Cutoff VAF ⩾ 0.5. 133 mutations not counted (VAF < 0.5). (a) Summary of gene alterations in 139 samples. (b) Percentage of gene alterations subtypes. (c) The most frequent gene alterations.

F1LA identified at least one clinical trial match not detected by standard diagnostic on 215 samples (53.9%) *versus* 25 samples (6.3%) on which only standard diagnostic had a clinical impact (Supplementary Table 2B). PCI with F1LA was 58.6% compared with 11.0% with standard diagnostic (RR = 5.32, 95% CI: 3.89–7.28, $p < 0.001$).

The median of number of clinical trials detected with F1LA increased when it was switched from F1L (median: 11; range: 0–48) to F1LCDx (median: 18; range: 0–64).

Focus on CRC

Since CRC subgroup had the greatest number of altered pathways, it was focused on CRC selected cases. In these cases, F1LA reported anti EGFR-resistant molecular alterations at the time of progression disease (Supplementary Table 3A–B). From 114 CRC F1LA, we selected 16 CRC samples with specific characteristics. They were BRAF, KRAS, and NRAS wild type at baseline on surgery tissue in local laboratory. Each F1LA was collected at radiological progression disease (PD) (RECIST 1.1). Moreover, each patient received at least one line of chemotherapy plus anti-EGFR before F1LA sampling.

Two patients showed amplifications of genes involved in EGFR acquired resistant mechanism (ERBB2 + GNAS or MET), all the other showed at least one EGFR acquired resistant mutation. Most frequent mutated genes included KRAS (10 out of 16 patients), EGFR and PIK3CA (both in 5 out of 16 patients), and BRAF (3 out of 16 patients). All patients showing EGFR mutation had at least one alteration in genes implicated in EGFR acquired resistant mechanisms.

Selected clinical cases

To demonstrate clinical utility and impact of F1LA, the most relevant cases were selected in which F1LA offered a therapeutic choice identifying crucial molecular alterations (Supplementary Table 4). Some of these patients were enrolled in a clinical trial or started a target therapy or an immunotherapy or an off-label therapy based on F1LA results.

Table 2. Pathways involved in the different subtypes of cancers.

Pathway	% of samples per pathway alterations						
	CRC (39 tot)	NSCLC(41 tot)	GC (13 tot)	PC (7 tot)	BTC (2 tot)	BC (10 tot)	Others (27 tot)
RAS	46.1%	9.7%	0%	28.6%	0%	10%	3.7%
WNT/APC	87.2%	2.4%	0%	14.3%	0%	0%	3.7%
HRR	33.3%	12.2%	15.4%	14.3%	0%	10%	3.7%
RTK	10.2%	9.4%	0%	0%	0%	0%	0%
PI3 K/PTEN/AKT/MTOR	25.6%	7.3%	23.1%	0%	0%	30%	11.1%
Hormone receptor	0%	0%	0%	0%	0%	40%	0%
MMR	2.6%	4.9%	0%	0%	0%	10%	0%
Apoptosis regulation	2.6%	0%	0%	0%	0%	0%	3.7%
Transcriptional regulation	20.5%	21.9%	7.7%	0%	0%	20%	18.5%
Cell-cycle regulation	2.6%	4.9%	7.7%	0%	0%	0%	3.7%
Chromatin remodeling	25.6%	34.1%	23.1%	14.3%	100%	30%	29.6%
Angiogenesis	2.6%	0%	7.7%	0%	0%	0%	0%
JAK/STAT	0%	2.4%	7.7%	0%	0%	0%	0%
TGF beta	20.5%	0%	0%	0%	0%	0%	3.7%
TP53	89.8%	34.1%	38.5%	14.3%	50%	20%	15.9%
Others	15.4%	17.1%	30.8%	0%	50%	0%	11.1%
RB	2.6%	4.9%	7.7%	14.3%	0%	10%	0%

Liquid CDx samples (324): 139 samples. BC, breast cancer; BTC, biliary tract cancer; CRC, colorectal cancer; GC, gastric cancer; NSCLC, non-small cell lung cancer; PC, pancreatic cancer.

100%–75%
74%–50%
49%–25%
24%–12.5%

In particular, two clinical cases in which F1LA radically changes the patient's clinical history are depicted in Figure 3(a) and (b).

In September 2019, an 82-year-old woman was diagnosed with stage IVa (cT2aN3M1b) lung adenocarcinoma reporting cough and dyspnoea without a prior history of smoking (Figure 3(a)).

The analysis performed on the cytology specimen resulted in wild type EGFR, negative ALK, negative Programmed deathligand-1 (PD-L1), and negative ROS1 (Figure 3(a)).

Based on the age, stage, and comorbidities, she started first-line mono-chemotherapy with gemcitabine at 1200 mg/mq day 1 and 8 every 3 weeks. Treatment with gemcitabine was poorly tolerated due to G2 anemia and intense asthenia resulting in 80% reduction of the total dose after the first cycle.

In January 2020, due to the worsening of respiratory symptoms, F1L was performed to investigate

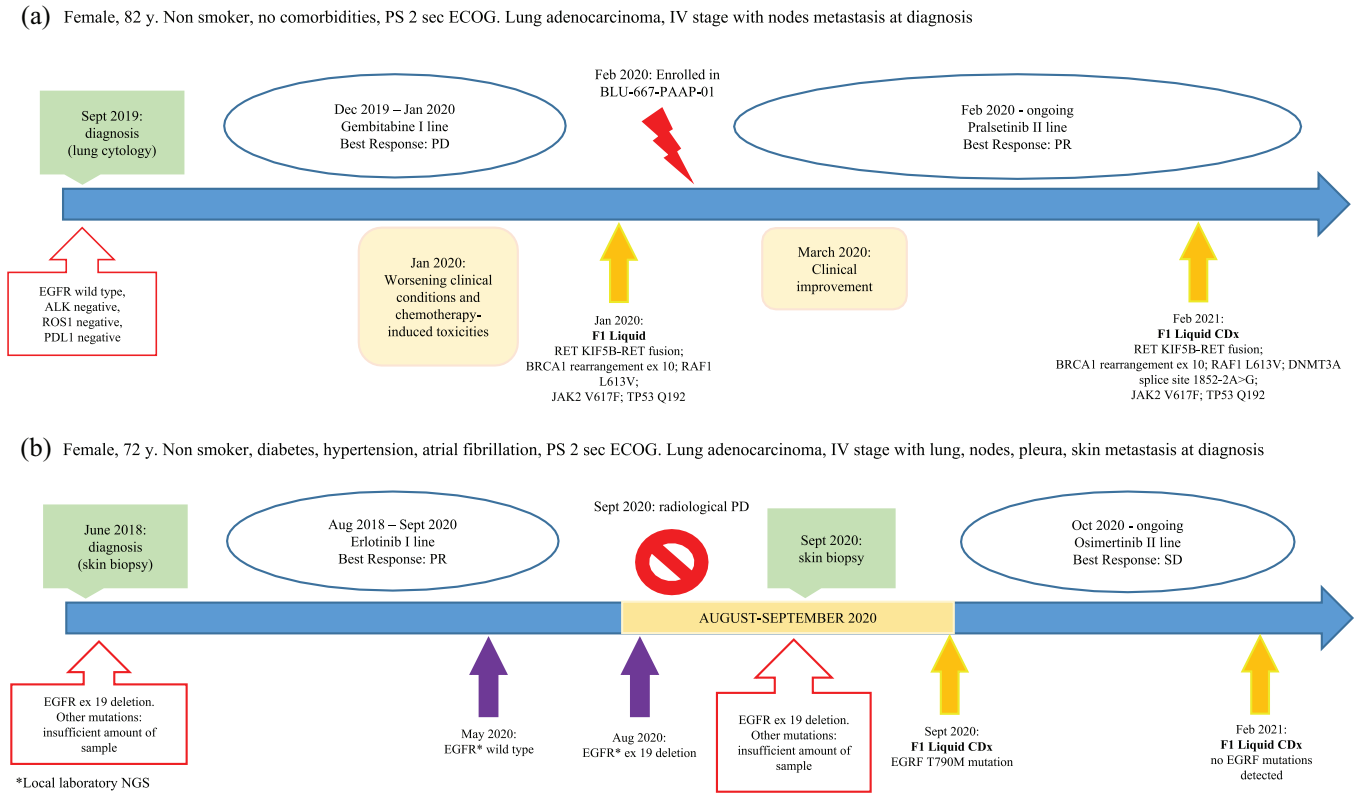

Figure 3. Selected cases in which Foundation Analysis changes the patient's clinical history. (a) Schematic representation of selected clinical case: the identification of KIF5B-RET fusion allowed target therapy. (b) Schematic representation of selected clinical case: the identification of EGFR T790M mutation allowed target therapy.

any potential druggable tumor alterations to offer therapeutic alternatives to the patient. Among the genes analyzed were detected KIF5B-RET fusion, BRCA1 rearrangement exon 10, RAF1 L613V mutation, JAK2 V617F mutation, and TP53 Q192 mutation.

Thus, considering both the poor tolerability of chemotherapy and worsening of clinical conditions, treatment with RET inhibitor pralsetinib was started at the dose of 400 mg once daily orally in February 2020.

After just 1 month of treatment, the patient showed an important clinical improvement reporting a progressive regression of symptoms (cough and dyspnoea) suggesting a rapid and dramatic response. In June 2020, total-body computed tomographic (CT) scan showed a partial response (PR) per RECIST criteria with reduction of the solid lesion in the upper lobe of the right lung, currently of about 20 × 16 mm *versus* 37 × 34 mm of the previous scan. A[18] F-FDG PET scan confirmed metabolic response in the right upper lung lobe (SUV 1.7 *versus* 15.6) and absence of lymph node uptake. The treatment was well tolerated with no significant drug-related adverse events (Figure 3(a)).

The Figure 3(b) illustrates a patient's (Patient 2, a female, 72 years old) poor performance status with cardiological and metabolic comorbidities. In 2018, she was diagnosed with stage IV lung adenocarcinoma with lung, pleura, nodes, and skin metastasis. At the diagnosis, only EGFR (exon 19 deletion) was tested because of insufficiency of the biopsy sample amount. She received erlotinib as first line from August 2018. In September 2020, CT scan revealed a radiological progression disease (PD) of all metastatic sites (Progression Free Survival PFS: 26 months). A biopsy on skin metastasis was performed. NGS revealed the persistence of EGFR alteration (exon 19 deletion). She was unfit for chemotherapy. At this time point, F1LCDx was performed. F1LCDx detected the resistant mutation T790M and allowed to start target therapy with osimertinib in October 2020. In February 2021, F1LCDx was repeated

and no molecular alterations of EGFR or resistant mutations were detected. The treatment is still ongoing, and patient achieved a significant improvement in performance status.

Discussion

The precision medicine era has revolutionized the way to make diagnosis, to monitor, and to treat each tumor based on its molecular profile. In this scenario, liquid biopsy appears to be a promising tool in clinical practice.[16]

In a previous work, we have evaluated the feasibility and clinical practice use of CGP performed with FoundationOne CDx assay (F1CDx) on tissue in a heterogeneous population of patients with metastatic cancer from our institution. After an analysis of success rate in either overall population and in different subgroups, we have evaluated whether the genomic alterations were relevant for each single patient.[17]

Based on the results obtained, in this work we have evaluated the clinical impact of F1LA on peripheral blood ctDNA from cancer patients treated in our institution. The study population was heterogeneous, including different tumor types, similar in age and gender distribution, underlying the clinical unmet need, partially overcame using F1LA.

F1LA is a comprehensive genomic profiling assay that could be a complement to tissue-based testing to evaluate potentially life-extending therapies for cancer patients. Additional data supporting the clinical validity of cfDNA-based testing is necessary to inform optimal use of these assays in the clinic.

F1LCDx assay is a pan-cancer cfDNA-based comprehensive genomic profiling assay that was recently approved by FDA.[14]

The transition of F1L to F1LCDx increased the success rate of the analysis to 99%, with improvement in sensitivity and specificity.[12,13] In our work, 97% of F1LCDx completed analysis (Supplementary Figure 1).

The majority of patients selected had PS 0-1 according to ECOG, both to ensure the best therapy immediately or before worsening clinical conditions and to allow them to be enrolled in clinical trials if targetable alterations were found. In particular, from patients' F1LA reports many actionable alterations and related clinical trials were highlighted. In addition, F1LA underlined target therapies approved and an analysis of the meaning of alterations for each tumor type was shown.[10,11,15]

Moreover, both pathological standard analysis on tissue in local laboratory and F1LA for each sample were performed. Compared with tissue analysis, the liquid biopsy allowed a heterogeneous characterization of the patient's molecular profile. F1LA drove a better diagnostic and therapeutic management of the patient in about three times the number of cases (RR = 2.94, 95% CI: 2.43–3.57, $p < 0.001$) and quintuples the possibility to identify the clinical trial match (RR = 5.32, 95% CI: 3.89–7.28, $p < 0.001$) (Supplementary Table 2).

These results suggest the integration of F1LA into clinical practice allowing a wider comprehension of patient's molecular profile. However, it should be noted that F1LA results interpretation and consequent clinical orientation were based on Multidisciplinary Molecular Tumor Board and on MOG, where patients were referred, rather than ESCAT (ESMO Scale for Clinical Actionability of molecular Targets) or OncoKB recommendations.

A crucial point was to determine the right time of performing F1LA. In our department, in a large number of other cancer, BTC, PC, GC, and NSCLC, F1LA was performed at the baseline of first line treatment; instead in a large number of CRC and breast cancer samples, F1LA was performed from the third line onward, according to the patients' cancer history.

For 40 out of 92 lung cancer patients, for 12 out of 24 pancreatic cancer patients and for 33 out of 58 other cancer patients, tissue biopsies were insufficient, not feasible or inadequate for testing mandatory biomarkers and another biopsy was not acceptable or safe for patient, thus the role of liquid biopsy has rapidly evolved as a valid alternative for initial genomic testing.[18–20] Liquid biopsy also offers chances to explore the underlying evolving tumor in a minimally invasive way to help inform cancer management.[21]

In addition, F1LA may be most appropriate for treatment response monitoring, and to identify genomic alterations indicative of response or resistance to therapy.[22–24] In particular, as we showed in Figure 3(b), F1LA overcomes tissue

and local NGS liquid assay limits in clinical applications.

Clonal evolution of disease and intratumor heterogeneity in response to selective pressures of different treatments represent a key challenge in cancer medicine. In this scenario, liquid biopsy may represent a tool able to recapitulate the overall tumor cell population in all different cancer subtypes (Supplementary Table 3).[25–36]

It must be noted there are limits in liquid biopsy use. First, actually many different commercial genomic panels are available and many NGS homemade assays are developed, each with different features, profiles, and costs. However, there is a lack of panels standardization, presence of confounder genomic information, and lack of appropriate clinical trial designs.[37,38]

Liquid biopsy is increasingly used as a noninvasive method for the genomic profiling of cancer and for evaluation of the patient at multiple time points. However, false-positive, and false-negative results are recognized as a major challenge that needs to be addressed.

False-positive results are associated with the several events including heterogeneity of the tumor, as well as DNA shed from normal cells.[39]

Genomic findings from cfDNA may originate from circulating tumor DNA fragments, germline alterations, or nontumor somatic alterations, such as clonal hematopoiesis of indeterminate potential (CHIP).[15,40,41] Recently, several papers identified false positive that could not be identified in tumor biopsy.[40] In particular, NGS of peripheral white blood cells revealed mutations derived from white blood cells (i.e. clonal hematopoiesis) and not from tumor. These findings underlined doubts regarding mutations detected in cfDNA, especially in clonal hematopoiesis-related genes, that may not always reflect tumor genotype.[40,41] Usually, clonal hematopoiesis of indeterminate potential is correlated with specific genes including, but not limited to, *DNMT3A*, *ASXL1*, and *TET2*, and, less commonly, *TP53*, *JAK2*, *NOTCH2*, *FAT3*, *EXT2*, *ERBB4*, *KRAS*, and *ARID2*.[41] The false-positive ctDNA due to the identification of molecular alterations linked to CHIP may determine an overestimation of the number of the positive liquid biopsies; interpretation should be based on clinical context. This could explain the high number of DNMT3A alterations (39) identified in the present work.

In addition, allelic frequencies and tumor heterogeneity have been associated with false-negative results, limiting the assay's ability to detect mutations. False negatives can occur if the amount of tumor DNA in the blood is so low that the percentage of mutated fragments is below the limit of detection.[39]

Thus, major efforts are needed to better address the false-positive and false-negative rates.

Selecting the right test at the right time for the right patient may be a challenge.[38,42] This decision may be influenced by many factors including patient and family expectations, emerging evidence, changing guidelines, clinical experience, and individual clinical, social, and cultural factors.

Our results demonstrate that F1LCDx accurately and reproducibly detects the major types of genomic alterations as well as complex biomarkers, such as MSI, bTMB, and TF. F1LCDx is actually the only one approved FDA companion test for many cancer types and many different genes.[43]

Recently, many studies demonstrated the undoubted benefits of liquid biopsy in clinical practice.[41,44–48] In addition, it must be noted the utility of liquid biopsy to detect an occult malignancy in patients with another cancer.[49]

Here, our data support the utility of F1LA in the oncological daily patient's approach. The test may be useful for diagnosis (as a complementary assay of tissue analysis), treatment choice in first and subsequent lines, therapy monitoring, residual disease assessing after surgery, for evaluating of therapy response or disease relapse and for evaluating prognosis (Supplementary Table 4).

F1LA provides to clinician a real-time genetic profile of cancer, including its heterogeneity in space and time. F1LA suggests the meaning of molecular alterations in each tumor type, target therapies approved, clinical trials available, updating this information constantly according to oncological knowledge.

Although additional studies are needed to validate the large use of liquid biopsy and F1LA, the comprehensive nature of F1LA demonstrates the reliability of test results providing genomic profiling results for cancer patients to be incorporated in clinical practice routine.

In conclusion, CGP with F1LA is a useful weapon for the clinician in real life oncological department setting. Further studies are needed to avoid overdiagnoses and to better select the patient type and the time in which it really will change the oncological future.

Ethics committee

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Supplemental material

Supplemental material for this article is available online.

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