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Mutational profiling using liquid biopsy to guide targeted therapy in patients with metastatic cancer

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Liquid biopsy gained significant interest in the area of cancer management. This study aims to evaluate the effectiveness of molecular testing using ctDNA (circulating tumor DNA) to; detect genetic alterations, screen for abnormalities, identify mutations associated with treatment sensitivity or resistance and guide therapy decision for several types of cancer in patients with metastasis. A total of 85 samples were collected from 74 patients recruited at our center, as part of their routine clinical follow-up. 17 different cancer types were analyzed. Genetic testing was conducted in patients with metastasis after failure of standard treatments. Sequencing was conducted in plasma-ctDNA samples; and when it was possible on the tumor tissue as well. Our analysis revealed that 88% (65 patients) of patients were eligible for treatment guidance using liquid biopsy. Among them, 64% (47 patients) received an FDA-approved drug, and treatment decisions were based on molecular testing using ctDNA. Somatic gene mutations were detected in 89% (66 patients) of the patients tested; 81% (60 patients) of patients had at least two mutations, 8% (6 patients) had only one mutation and 11% (8 patients) had no detected mutations. Interestingly, among the genes tested, BRCA2, EGFR, MSH6, and NF1 were the most frequently mutated in our patients. Our study highlights the potential benefits of personalized medicine through a non-invasive genetic testing across patients with metastasis regardless of the cancer types. Moreover, our study identified the frequent occurrence of specific gene mutations across various types of cancer, which paves the way for considering targeted therapies that could be applicable to multiple cancer types, rather than being restricted to just a few.

Keywords Liquid biopsy, Cancers, Treatment guidance, Metastasis

For decades, tumor biopsy has been the gold standard for cancer diagnosis. This approach, however, presents several challenges and is not suitable for routine monitoring of cancer progression, since solid biopsy cannot be performed repeatedly to track molecular changes during disease progression and drug resistance, which is crucial for guiding treatment decisions[1,2]. Drug resistance is a significant issue in cancer treatment, with recent studies indicating that 90% of chemotherapy failures are due to drug resistance that develops during disease invasion and metastasis. As chemotherapy is administered, many tumor cells become resistant through various molecular mechanisms, including drug inactivation, epigenetic changes, and inhibition of cell death[3]. In this context, tumor heterogeneity plays a pivotal role in treatment response. By using tumor biopsy at one site, it gives tumor snapshot only at a single location, and it could ignore spatial tumor heterogeneity, which is often observed in patients[1,4]. Unlike solid biopsy, liquid biopsy particularly those involving circulating tumor DNA (ctDNA) from plasma have recently gained significant interest in the detection of genomic alterations and the development of precision therapies[5]. Extensive research has demonstrated that liquid biopsy offers numerous clinical advantages. It provides anatomically unbiased sampling of both primary and metastatic tumor lesions[6] and can reveal the molecular status of cancers at any stage of the disease[7], aiding oncologists in identifying molecular abnormalities that drive cancer initiation. This valuable information enables informed treatment decisions to be made and allows for the monitoring of patient response to therapy[2,8]. The advent of liquid biopsy has made it possible to perform comprehensive tumor genomic profiling, which supports oncologists in making more accurate treatment recommendations and ensuring that the appropriate targeted therapy is administered to the right patient[9].

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In today's era of precision medicine, liquid biopsy plays a crucial role in evaluating the genetic aberrations that drive the initiation and progression of tumor cells, enabling the selection of appropriate targeted therapies for patients (1). Many gene mutations are effectively targeted in precision medicine using various drugs, providing new opportunities for personalized treatment. Targeted therapies are increasingly being integrated into clinical care, thanks to technological advancements that allow for rapid and cost-effective genetic detection. Among these technologies, Next-Generation Sequencing (NGS) is a stable and excellent technology capable of analyzing millions of DNA fragments simultaneously[10]. NGS technology is used to detect mutations at any stage of the disease, targeting specific genes or regulatory regions in ctDNA by selecting genomic areas of interest[11]. This approach ensures comprehensive genomic analysis, allowing for the detection of various types of genetic alterations with high sensitivity, greater speed, and at a much lower cost[12].

In this paper we evaluate, using real world data in different types of cancer, the significance of using molecular testing based on ctDNA from plasma samples employing NGS to detect genetic alterations in some of the most prevalent oncology-related genes. By understanding the strengths and the limitations of using these tests in identifying actionable mutations and variants associated with treatment sensitivity and/or resistance, we aim to provide specialists and physicians with useful insights that will help them manage their patients' treatment and follow-up.

Moreover, our study identified several classes of mutation variants that meet the evidence threshold for causality or contribution to cancer, even when the tests used are restricted to a limited number of genes. Additionally, we observed the frequent occurrence of specific gene mutations across various cancer types, which pave the way for considering targeted therapies that could be broadly applicable, rather than limited to only a few cancer types.

Materials and methods

Study design

This descriptive cross-sectional study was designed to analyze the clinicopathologic and genomic data of 74 patients with diverse solid cancers, who were followed et al. Azhar Oncology Center in Rabat, Morocco. The study focused on patients for whom molecular testing using circulating tumor DNA (ctDNA) had been performed on plasma samples, and, when possible, on FFPE tissues. Molecular testing was ordered by the treating physicians for patients with advanced cancer to identify clinically relevant genomic alterations that could potentially guide treatment decisions. All patients undergoing molecular testing using ctDNA signed a clinical consent form provided by the laboratory. In this study the tests were done for clinical purpose and the patients provided written informed consent to have their genomic data published anonymously. The study was authorized by the Al Azhar Oncology Center board.

Patients

Seventy-four patients with cancer were recruited at the Al Azhar Oncology center in Rabat, Morocco, resulting in 17 groups of various solid cancers. These cancers included breast cancer, lung cancer, colorectal cancer, pancreatic cancer, sarcoma, kidney cancer, gastric cancer, glioblastoma, adenoid cystic cancer, urothelial cancer, desmoid cancer, cervix cancer, Ewing cancer, adrenocortical cancer, endometrial cancer, liver cancer and brain cancer. As part of their routine clinical follow-up, genetic tests were conducted for patients with metastatic cancer who had not responded to standard first-line curative or metastatic treatments, regardless of tumor location, in order to identify actionable mutations that might explain the failure of conventional treatments and to explore the potential for targeted therapies. Molecular testing was primarily performed on plasma samples, and when possible, on tumor tissue as well.

Molecular testing

Sampling and DNA extraction

20 ml of blood were collected from each patient into Streck Cell-Free DNA BCT tubes to prevent contamination of circulating tumor DNA (ctDNA) by genomic DNA (gDNA). All samples were centrifuged at room temperature to separate the plasma. The centrifugation was performed in two stages: first at 1000 rpm for 10 min, followed by a second centrifugation at 6,000 rpm for 10 min. The plasma was either used immediately for DNA extraction or stored at -80°C for future use. Microdissections from formalin-fixed paraffin-embedded (FFPE) specimens of tissue biopsies were performed. Only tissue areas with at least 20% of tumor cells were used for gDNA extraction. This threshold ensures that the DNA extracted is predominantly from tumor cells which minimizing contamination from non-tumor cells. gDNA was extracted from formalin-fixed paraffin-embedded (FFPE) specimens using the PureLinkTM Genomic DNA mini kit (Invitrogen by Thermo Fisher Scientific) following manufacturer's instructions. ctDNA was isolated from plasma samples using QIAamp® Circulating Nucleic Acid kit (Qiagen, Germany) following manufacturer's instructions. DNA extracts was controlled quantitatively by Nanophotometer (IMPLEN Nanophotometer P-330) and Qubit 3.0 fluorometer (Life technologies). The ctDNA and gDNA used either immediately for library preparation for sequencing or stored at -20°C until use.

All experimental protocols were approved by the FPN institution, Clinical Pathology laboratory and Al Azhar Oncology Center. All experiments were performed in accordance with relevant guidelines and regulations and informed consent was obtained from all participants and/or their legal guardians.

Library preparation and sequencing

10 ng of extracted DNA was subjected to library preparation followed by next-generation sequencing using the Illumina NextSeq technology. The majority of the libraries were prepared using OptiSeq^{tm} NGS Pan-Cancer Panel Service (Diacarta, CA, USA) following manufacturer's instructions. Cleanup was performed using SPRIselect

beads (Beckman; CA, USA) following manufacturer's instructions. Next-generation sequencing was performed using the NextSeq 500 MID Output Flow cell (Illumina; CA, USA) following manufacturer's instructions.

Pair-end deep sequencing (>100X) was conducted on cancer gene panel using targeted next-generation sequencing assays. The study utilized various assay types, namely: Panel 1, Panel 2, Panel 3, Panel 4, Panel 5 and Panel 6 covering respectively 11, 26, 35, 56, 65, and 105 genes (Table 1). These tests cover all the gene sequence, including intronic and exonic sequences.

Among the 85 samples collected from 74 patients, 54 samples (63.5%) underwent the Panel-5 test (65 genes), 9 samples (10.6%) had the Panel-6 test (105 genes), 7 samples (8.2%) had the Panel-4 test (56 genes), 5 samples (5.9%) had the Panel-1 test (11 genes), 4 samples (4.7%) had the Panel-3 test (35 genes), and 4 samples (4.7%) had the Panel-2 test (26 genes). Additionally, 2 samples (2.4%) underwent whole genome sequencing. Sequencing was conducted on either tumor tissue or plasma samples. Among the 74 patients, 54 (72.97%) had plasma samples tested, 14 (18.92%) had FFPE tissue tested, and 6 (8.11%) had both types of samples tested, with 2 patients undergoing more than two tests for disease follow-up.

Data analysis

The data analysis was performed using Qiagen CLC Genomics Workbench (Qiagen). The sequencing reads were filtered to include only those with a Q-score above 20, the reads were aligned to the human reference genome (GRCh37/hg19) using the Burrows-Wheeler Aligner (BWA). Duplicate reads, resulting from clonal amplification of the same fragments during library construction, were removed, as were regions with mapping coverage of less than 50×. Variant detection analysis was performed to identify SNPs, insertions, and deletions. Only high-confidence variants were retained according to the following criteria: (1) occurring in a high-complexity region, (2) having high, or higher than expected, variant fraction (germline), (3) having a sequencing coverage > 50×, (4) having > 50 supporting reads, and (5) being aligned inside the target region. Variant annotation details, including coding consequences, were considered for further analysis. For each variant, we reported the variant frequency, calculated as the variation percent = (altNum/depth) × 100, where "altNum" represents the number of reads containing the variant and "depth" is the total number of reads covering both the variant and the reference allele. The variants were annotated and filtered using the Golden Helix VarSeq analysis workflow implementing the AMP guidelines for interpretation of sequence variants. This includes comparison against the genomADpopulation catalog of variants in 123,136 exomes, the 1000 Genomes Project Consortium's publication of 2,500 genomes, the NCBI ClinVar database of clinical assertions on variant's pathogenicity and multiple lines of computational evidence on conservation and functional impact. The following databases and algorithms are used to annotate and evaluate the impact of the variant in the context of human disease: 1000 genomes, gnomAD, ClinVar, OMIM, dbSNP, NCIB RefSeq Genes, ExAC Gene Constraints, VS-SIFT, VS-PolyPhen2, PhyloP, GERP + +, GeneSplicer, MaxEntScan, NNSplice, PWM Splice Predictor. Analysis was reported using HGVS nomenclature (www.hgvs.org/mutnomen) as implemented by the VarSeq transcript annotation algorithm. The statistical analysis conducted in this study involved basic descriptive and inferential statistics, such as mean comparisons, standard deviations, and simple hypothesis testing.

Results

Patients' characteristics

Circulating tumor DNA molecular testing was performed, reviewed, and analyzed for 74 patients (Table 2). The difference in gender proportion, with females representing 55.4% (41 female) and males 44.6% (33 male), was not significant, as we assessed by performing a 1-sample proportions test with continuity correction against the null hypothesis that the proportion was 50% and obtaining a p-value > 0.05. The median age of the patients was 59

Test name	Genes detected	NGS tool
Panel 1	ALK/BRAF/EGFR/ERBB2/KRAS/MAP2K1/MET/NRAS/PIK3CA/ROS1/TP53	Illumina
Panel 2	ALK/PTEN/PIK3R1/EGFR/NRAS/STK11/ERBB4/ERBB2/AKT1/HIST1H3B/HRAS/PDGFRA/MAP2K1/CDKN2A/IDH1/H3F3A/IDH2/BRAF/PIK3CA/ KIT/CTNNB1/KRAS/FGFR3/MET/FGFR2/DDR2	Illumina
Panel 3	AKT1/ALK/AR/ BRAF/CDK4/CTNNB1/DDR2/EGFR/ERBB2/ERBB3/ERBB4/ESR1/FGFR2/ FGFR3/GNA11/GNAQ/HRAS/IDH1/IDH2 /JAK1/JAK2/JAK3/ KIT/KRAS /MAP2K1/ MAP2K2/MET/MTOR/NRAS/PDGFRA/PIK3CA/RAF1/RET/ROS1/SMO	Illumina
Panel 4	ABL1/AKT1/ALK/APC/ATM/BRAF/CDH1/CDKN2A/CSF1R/CTNNB1/DDR2 /DNMT3A/EGFR/ERBB2/ERBB4/EZH2/FBXW7/FGFR1/FGFR2 /FGFR3/ FLT3/FOXL2/GNA11/GNAQ/GNAS/HNF1A/HRAS/IDH1/IDH2/JAK2/JAK3/KDR/KIT/KRAS/MAP2K1/MET/MLH1/MPL/MSH6/NOTCH1/NPM1/ NRAS/PDGFRA/ PIK3CA/PTEN/PTPN11/RB1/RET/SMAD4/SMARCB1/SMO/SRC/STK11/TP53/TSC1/VHL	Illumina
Panel 5	ABL1/NF1/FGFR1/IDH1/MPL/FLT3/DNMT3A/APC/CTNNB1/FGFR2/IDH2/MSH6/AKT1/CSF1R/ALK/DDR2 /FGFR3/JAK2/MTOR/JAK3/PIK3R1/ STK11/PTCH1/TERT/PTEN/TP53/PTPN11/TSC1/KDR/VHL/ATM/NF2/RB1/EGFR/FOXL2/GNA11/KIT/BRAF/ERBB2/NOTCH1/RET/SMAD4/NPM1/ KRAS/GNAQ/BRCA1/ERBB3/SMARCB1/BRCA2/ERBB4/NRAS/GNAS/MAP2K1/SMO/EZH2/MET/PDGFRA/HNF1A/CDH1/HRAS/CDKN2A/FBXW7/ PIK3CA/SRC/MLH1	Illumina
Panel 6	AKT1/AKT2/ALK/APC/AR/ARAF/ARID1A/ATM/ATR/B2M/BAP1/BRAF/BRCA1/BRCA2/BTK/CCND1/CCND2/CCND3/CCNE1/CD274/PD-L1/CDH1/ CDK4/CDK6/CDKN2A/CTNNB1/ DDR2, DPYD, EGFR, ERBB2/HER2/ERRFI1/ESR1/EZH2/FBXW7/FGFR1/FGFR2/FGFR3/FGFR4/FLT3/FOXL2/GATA3/ GNA11/GNAQ/GNAS/HNF1A/HRAS/IDH1/IDH2/JAK1/JAK2/JAK3/KDR/KEAP1/KIT/KMT2A/KRAS/MAP2K1/MAP2K2/MAPK1/MET/MLH1/MPL/ MSH2/MSH3/MSH6/MTOR/MYC/MYCN/NF1/NF2/NFE2L2/NOTCH1/NPM1/NRAS/NTRK1/PALB2/PBRM1/PDCD1LG2/PDGFRA/PDGFRB/PIK3CA/ PIK3R1/PMS2/PTCH1/PTEN/PTPN11/RAD51C/RAF1/RB1/RET/RHEB/RHOA/RIT1/RNF43/ROS1/SDHA/SMAD4/SMO/SPOP/STK11/TERT/TP53/TSC1/ TSC2/UGT1A1/VHL/ Gene Rearrangements /ALK/BRAF/FGFR2/FGFR3/NTRK1/RET/ROS1/Copy Number Gains/CCNE1/CD274/PD-L1/EGFR/ERBB2/ HER2/MET/MYC/Copy Number Gains Losses/BRCA1/BRCA2	Illumina

Table 1. Genes identified in tests used in the study.

Characteristics	Patients
Total patients (N) Age (years)	74
Median age (Interquartile range)	59 (21.00)
Range	11–89
Patients between 18 & 40 (N, %)	15 (20.28%)
Patients between 40 & 89 (N, %)	55 (74.32%)
Not provided (N, %)	4 (5.40%)
Gender (N, %)	
Male	33 (44.60)
Female	41 (55.40)
Type of cancer (N, %)	
Breast cancer	17 (22.97%)
Lung cancer	13 (17.56%)
Colorectal cancer	10 (13.52%)
Pancreatic cancer	6 (8.11%)
Sarcoma	6 (8.11%)
Kidney cancer	2 (2.70%)
Gastric cancer	2 (2.70%)
Unknown tumor	8 (10.81%)
Other*	10 (13.52%)
Sample type (N, %)	
Liquid biopsy	54 (72.97%)
Tissue biopsy	14 (18.92%)
Both spicimens	6 (8.11%)
Age at diagnosis (years)	
Median (Range/Interquartile range)	52 (10–78/14.75)
Treated already with conventional therapy (N, %)	
Chemotherapy	6 (8.11%)
Radiotherapy	1 (1.35%)
Surgery/Chemotherapy	11 (14.87%)
Surgery/Radiotherapy	1 (1.35%)
Chemotherapy/Radiotherapy	7 (9.46%)
All	17 (22.97%)
Not provided	31 (41.89%)
Treated with targeted therapy before genetic test	0 (0%)

Table 2. Patient characteristics. *Glioblastoma, Ewing cancer, adenoid cystic cancer, urothelial cancer, adrenocortical cancer, endometrial cancer, desmoid tumor, cervix cancer, liver cancer, brain cancer (each N = 1).

years, the ages of the patients ranged from 11 to 89 years, with a standard deviation of 16.35 and an interquartile range of 21, while the median age at tumor diagnosis was 52 years, the age at tumor diagnosis ranged from 10 to 78 years, with a standard deviation of 16.01 and an interquartile range of 14.75. Among the 74 patients, 17 different cancer types were analyzed. The most common cancer was breast (17 patients, 22.97%), followed by lung (13 patients, 17.56%), colorectal (10 patients, 13.52%), pancreatic (6 patients, 8.11%), sarcoma (6 patients, 8.11%), kidney (2 patients, 2.70%), and gastric (2 patients, 2.70%). Other cancer types each represented a single patient (Table 2). Additionally, 8 patients (10.81%) had metastatic cancer with an unknown tumor origin.

At the time of testing all patients presented with at least one metastatic site and they had failed to respond to standard first-line curative or metastatic treatments, as recommended by the official clinical guidelines, and the physicians had ordered molecular genetic testing to identify aberrations leading to tumor resistance, determine alternative FDA-approved targeted treatments, and understand alterations contributing to drug escape and resistance. Prior to molecular testing, 17 patients (22.97%) had received chemotherapy, surgery, and radiotherapy, 11 patients (14.87%) had undergone surgery and chemotherapy, 7 patients (9.46%) had received chemotherapy and radiotherapy, 6 patients (8.11%) had been treated with chemotherapy only, 1 patient (1.35%) had received radiotherapy only, and 1 patient (1.35%) had undergone surgery and radiotherapy. No patients had been treated with targeted therapy before the genetic test.

Tissue biopsy & liquid biopsy technical concordance

From the analysis of our results, we found that liquid biopsy data closely resemble those obtained from tissue biopsy in terms of DNA library quality control, data throughput, data quality, bioinformatics analysis, and

genetic alteration detection. Libraries were prepared, sequenced, and showed a peak at the expected size of approximately 300 bp (Fig. 1A). The total quantity of the library ranged from 1.34 ng/µl to 35 ng/µl, consistent with the company's recommendations for library preparation. All libraries were successfully sequenced, generating sufficient reads per sample. Of the total reads generated, approximately 98% were merged. Among the merged reads, 98% were successfully mapped, and 96% were trimmed (Fig. 1B). Regarding genetic alteration detection, among the 6 patients (8.11%) who underwent both ctDNA and tissue genetic testing, 3 patients had common alterations detected in both FFPE-tissue and matched liquid biopsy samples. The remaining 3 patients had genetic alterations detected only in their liquid biopsy samples and not in their FFPE-tissues (Supplementary material, Table 1).

Changes in the mutational profile for patients who underwent more than one liquid biopsy test

The analysis of the mutation profiles in patient who underwent at least two ctDNA testing (two patients) reveals significant temporal changes across the samples (Table 3), (Supplementary material, Table 1). For patient P4, we have three liquid biopsy samples (55, 11 and 4) performed in 19/09/2019, 17/12/2019 and 19/05/2020, respectively. Diverse mutations were detected in sample 55 indicating a heterogeneous tumor population. Sample 11 conducted later, showed persistence of 1 mutation and two mutations were developed, while others mutations were no longer detected. Notably, in the latest liquid biopsy of Sample 4, we found new mutations in BRCA2, MSH6 and NOTCH1 suggesting clonal evolution.

For patient 12, two samples 37 and 15, corresponding to liquid biopsies collected in 21/05/2018 and 14/10/2019, respectively. The Sample 37 displays additional mutations, such as RET (c.2307G > T) and SMAD4 (c.1060T > C), compared to tissue biopsy and conversely, tissue biopsy contains mutations that are not observed in sample 37. Samples 15 was collected more than a year later, revealing the emergence of a new mutation in KRAS (c.34G > C), while other mutations detected previously were no longer present.

Gene alterations analysis

NGS enabled us to identify the mutational profiles of various genes. We detected somatic gene mutations in 89% (66 patients) of the patients tested, while 11% (8 patients) either had no detectable ctDNA in their plasma or had ctDNA present but with no mutations identified (Fig. 2). In our cohort, 88% (65 patients) of patients were eligible for treatment guidance based on liquid biopsy results: 64% (47 patients) were matched with available FDA-approved drugs, 9% (7 patients) were potential candidates for clinical trials involving experimental drugs, and 15% (11 patients) were advised to avoid certain drugs associated with tumor resistance. Only 1% (1 patient) of patients had non-actionable mutations, meaning they had no available drug options or applicable clinical trials.

NGS allowed us to identify a range of somatic genetic alterations in our cohort. We found that 8% (7/85) of samples had only one mutation, 81% (69/85) had at least two mutations, and 11% (9/85) had no detectable mutations. These findings underscore the complexity of tumor development and progression. While a reductionist approach may be suitable for a small fraction of patients harboring one actionable mutation, a

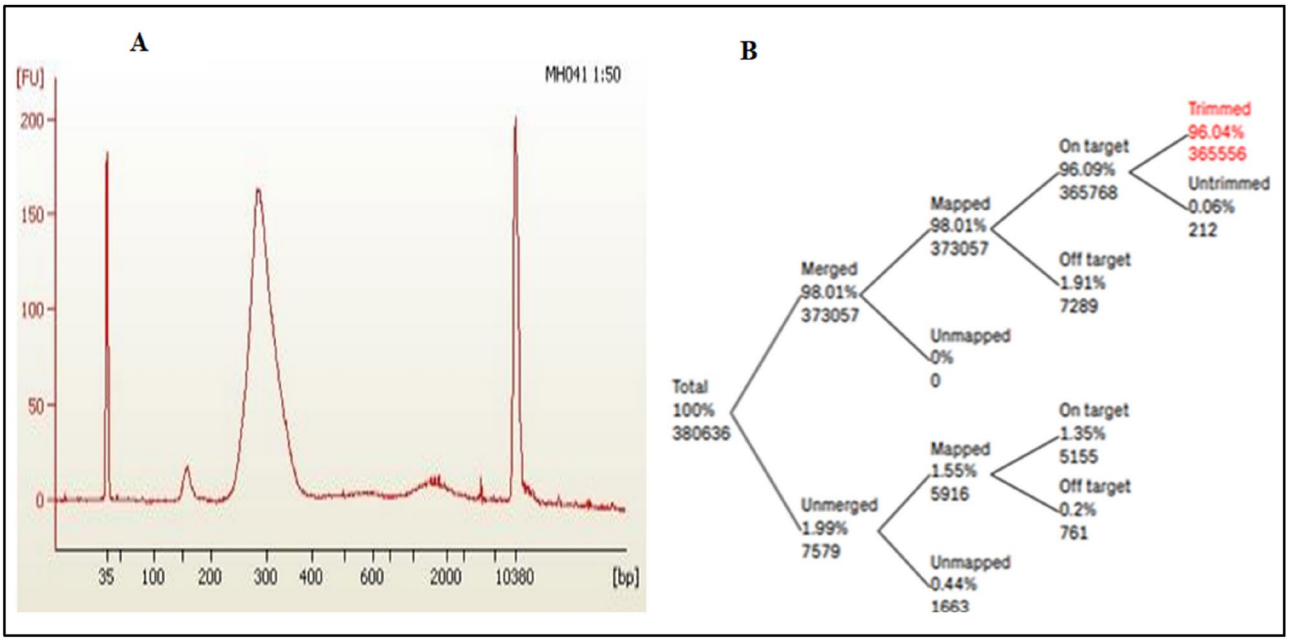

Fig. 1. (**A**) Bioanalyzer trace representing size of an example of sequencing library for a patient (MH041). Sequenced library showed peak at expected size ~ 300 bp. (**B**) patient example of mapping statistics . All libraries were sequenced and sufficient reads were generated per sample.

Patient ID	Sample ID	Date of First treatment received	Sample type	Collection date
P1	1	01/2021	Tissue biopsy	01/02/2021
P2	2	04/2019	Tissue biopsy	12/2020
P3	3	–	Liquid biopsy	23/06/2020
	23		Liquid biopsy	31/01/2018
P4	4	02/2019	Liquid biopsy	19/05/2020
	11		Liquid biopsy	17/12/2019
	12		Tissue Biopsy	09/2019
	55		Liquid biopsy	19/09/2019
P5	5	09/2019	Liquid biopsy	28/04/2020
P6	6	02/2019	Liquid biopsy	28/04/2020
P7	7	02/2018	Tissue biopsy	16/09/2020
P8	8	02/2020	Liquid biopsy	03/03/2020
P9	9	01/2019	Liquid biopsy	04/03/2020
P10	10	04/2017	Tissue biopsy	27/11/2019
P11	13	–	Liquid biopsy	16/06/2020
P12	14	–	Tissue biopsy	10/2019
	15		Liquid biopsy	14/10/2019
	37		Liquid biopsy	21/05/2018
	38		Tissue biopsy	08/05/2018
P13	16	–	Liquid biopsy	15/01/2020
P14	17	–	Liquid biopsy	18/12/2019
P15	18	–	Liquid biopsy	03/2018
P16	19	10/2017	Liquid biopsy	10/04/2018
P17	20	06/2016	Liquid biopsy	02/ 2018
P18	21	–	Liquid biopsy	03/04/2018
P19	22	12/2015	Tissue biopsy	04/2018
P20	24	01/2018	Liquid biopsy	03/2018
P21	25	09/2019	Liquid biopsy	28/11/2019
P22	26	2016	Liquid biopsy	12/07/2018
P23	27	–	Tissue biopsy	11/09/2018
P24	28	02/2017	Tissue biopsy	14/09/2018
	51		Liquid biopsy	20/09/2018
P25	29	–	Liquid biospy	13/06/2018
P26	30	–	Liquid biopsy	29/03/2021
P27	31	01/2017	Liquid biopsy	13/07/2021
P28	32	–	Liquid biopsy	06/2021
P29	33	2017	Liquid biopsy	09/08/2021
P30	34	–	Tissue biopsy	13/07/2021
P31	35	2018	Liquid biopsy	09/06/2021
P32	36	11/2019	Liquid biopsy	25/10/2021
P33	39	–	Liquid biopsy	04/04/2018
P34	40	12/2016	Tissue biopsy	12/01/2017
	41		Liquid biopsy	12/01/2017
P35	42	–	Tissue biopsy	12/01/2017
P36	43	04/2012	Liquid biopsy	16/03/2018
P37	44	–	Liquid biopsy	05/11/2017
	45		Tissue biopsy	11/10/2017
P38	46	02/2015	Liquid biopsy	05/04/2018
P39	47	12/2016	Liquid biopsy	15/02/2018
P40	48	–	Tissue biopsy	03/06/2019
P41	49	12/2014	Tissue biopsy	01/07/2019
P42	50	–	Liquid biopsy	02/08/2021
P43	52	–	Liquid biopsy	29/11/2019
P44	53	–	Liquid biopsy	01/03/2021
P45	54	–	Liquid biopsy	25/05/2021

Continued

Patient ID	Sample ID	Date of First treatment received	Sample type	Collection date
P46	56	07/2020	Tissue biopsy	11/2021
	57		Liquid biopsy	11/2021
P47	58	03/2018	Liquid biopsy	06/12/2021
P48	59	12/2016	Liquid biopsy	11/11/2021
P49	60	07/2020	Liquid biopsy	27/12/2021
P50	61	–	Liquid biopsy	07/02/2022
P51	62	05/2021	Liquid biopsy	21/12/2021
P52	63	06/2021	Liquid biopsy	27/12/2021
P53	64	–	Tissue biopsy	03/2022
P54	65	–	Liquid biopsy	11/04/2022
P55	66	–	Liquid biopsy	21/03/2022
P56	67	–	Liquid biopsy	11/04/2022
P57	68	–	Liquid biopsy	18/04/2022
P58	69	10/2019	Liquid biopsy	16/05/2022
P59	70	12/2021	Liquid biopsy	30/05/2022
P60	71	–	Liquid biopsy	09/05/2022
P61	72	2019	Liquid biopsy	06/06/2022
P62	73	–	Liquid biopsy	01/05/2022
P63	74	–	Liquid biopsy	10/04/2022
P64	75	07/2022	Tissue biopsy	06/06/2022
P65	76	07/2022	Tissue biopsy	12/07/2022
P66	77	09/2022	Liquid biopsy	27/03/2022
P67	78	2022	Tissue biopsy	27/09/2022
P68	79	2018	Liquid biopsy	06/06/2022
P69	80	06/2022	Liquid biopsy	12/07/2022
P70	81	–	Liquid biopsy	16/05/2022
P71	82	–	Liquid biopsy	25/06/2022
P72	83	03/2023	Liquid biopsy	14/03/2023
P73	84	–	Liquid biopsy	23/01/2023
P74	85	02/2023	Liquid biopsy	03/05/2023

Table 3. Dates of biopsy collection.

systemic analysis approach is necessary for the effective follow-up and management of most patients which harbor several mutations.

In our analysis of 71 genes, we identified 353 distinct genetic alterations, which were classified into five categories: deletions (47%), transitions (30%), transversions (11%), duplications (11%), and deletion/insertion events (1%) (Fig. 3A). By analyzing the proportions of these mutations, we found that only six cancer types namely; breast cancer, lung cancer, colorectal cancer, pancreatic cancer, gastric cancer and cervix cancer present all the aberrations groups, six cancers namely; sarcoma, glioblastoma, kidney cancer, adenoid cystic cancer endometrial cancer and desmoid cancer had 4 mutations types, urothelial cancer and liver cancer had 3 mutations groups, only 2 mutations type were observed in Ewing cancer and brain cancer and no mutation was detected in adrenocortical cancer patient (Fig. 3B). Regarding the frequency of mutated genes detected in our cohort we observed that BRCA2, EGFR2, MSH6 and NF1 genes were the most mutated genes in the 17 cancer types investigated (Fig. 4).

Mutations profile

The following 10 genes were found to be altered in more than 10 cancer types : BRCA2, EGFR, NF1, MSH6, PTEN, APC, ATM, PDGFRA, NOTCH1, FGFR1. Among these, the BRCA2 gene mutations found in the highest proportion of samples observed with 5 genetic alterations frequently detected. Other gene mutations had been found in less than 9 cancer types including mutations in the following genes: TP53, PIK3CA, RB1, PTCH1, ALK, FGFR3, NPM1, RET, MET, HRAS, IDH1, KDR, CSF1R, BRCA1, KRAS, STK11, FLT3, NF2, KIT, HIST1H3B, HNF1A, CDKN2A, DNMT3A, JAK3, SMARCB1, GNA11, ABL1, BRAF, DDR2, MSH2, NRAS, KMT2D, SMAD4, PMS2, MSH3, MLH3, FGFR2, MAP2K1, TERT, PMS1, AKT1, MTOR, POLE, FBXW7, RNF43, ARID1A, PTPN11, CDH1, MPL, ERBB3, ERBB4, CTNNB1, VHL, JAK2, PD-L1, ROS1, KMT2C, EZH2, GNAS, ZFHX3 and SMO (Fig. 4).

Mutations associated with drug sensitivity

DNA sequencing has been used routinely to inform the choice of targeted therapy in specific cancer type. Genetic alterations associated with sensitivity to drugs across tumor types were observed in several genes. Of

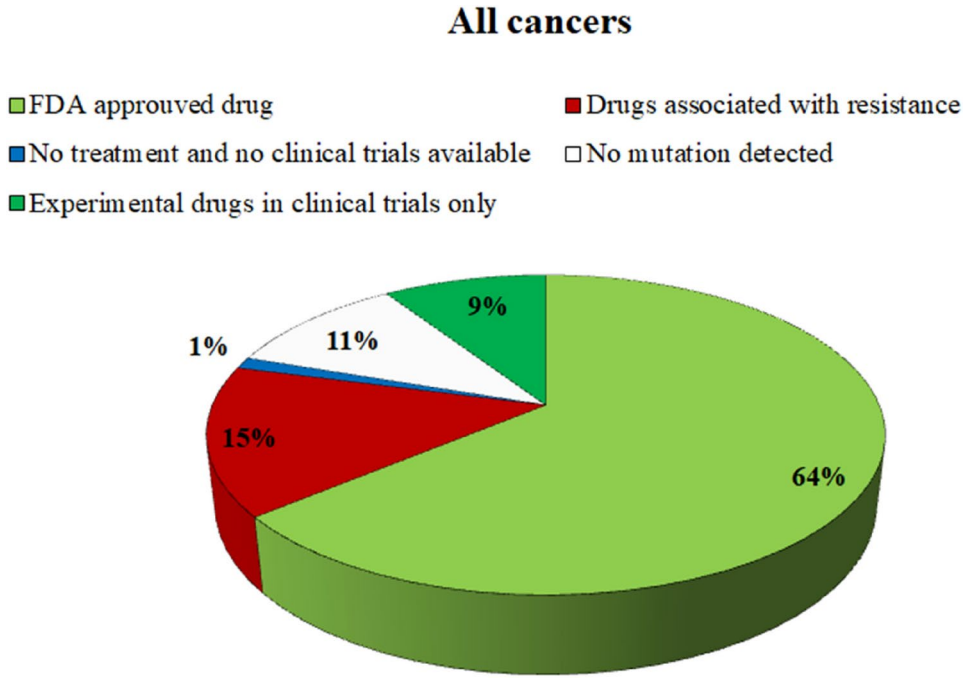

Fig. 2. Pie chart representing the percentage of patients who benefits or not from treatment guidance based on genetic profiling of each patient. 88% (65 patients) benefits from treatment guidance (64% (47 patients) with FDA-approved drug, 9% (7 patients) could join experimental drugs in clinical trials and 15% (11 patients) avoided drug associated with resistance). 11% (8 patients) of patients registered either with no ctDNA in their plasma or, with ctDNA but with no detected mutations no detected mutations and 1% (1 patient) of patients with detected mutation but without available drug or clinical trial).

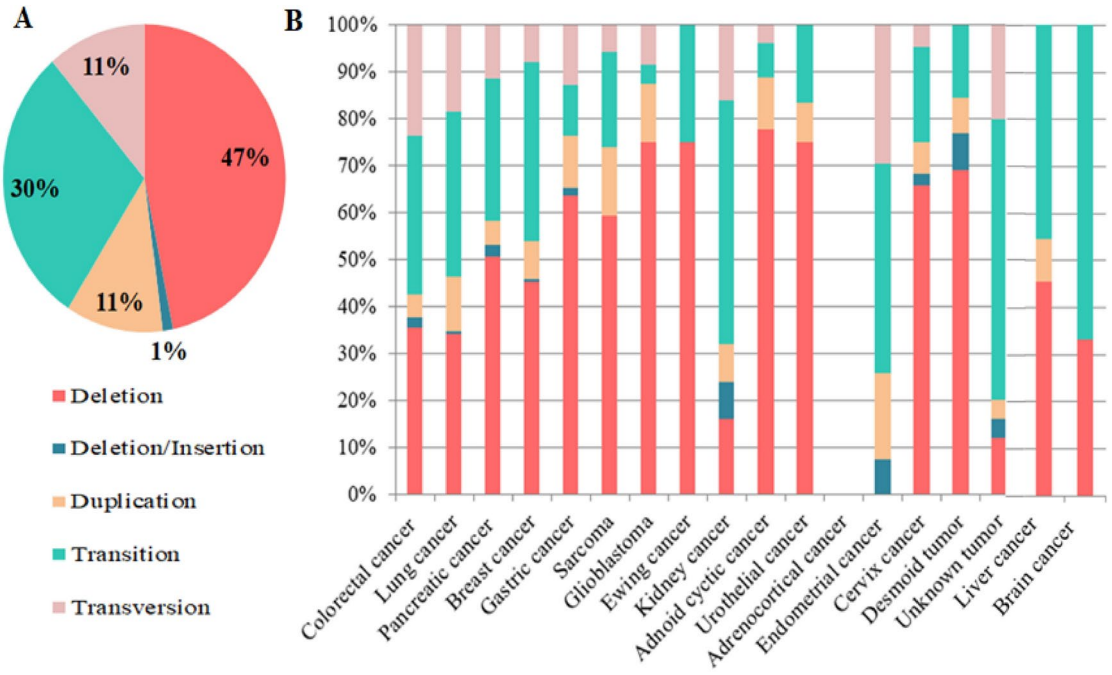

Fig. 3. (**A**) Pie graph representing the proportions of mutations classes in our cohort, we found deletions (47%), transitions (30%), transversions (11%) duplications (11%) and deletion/insertion (1%). (**B**) Bar graph representing the proportions of mutations in each type of cancer.

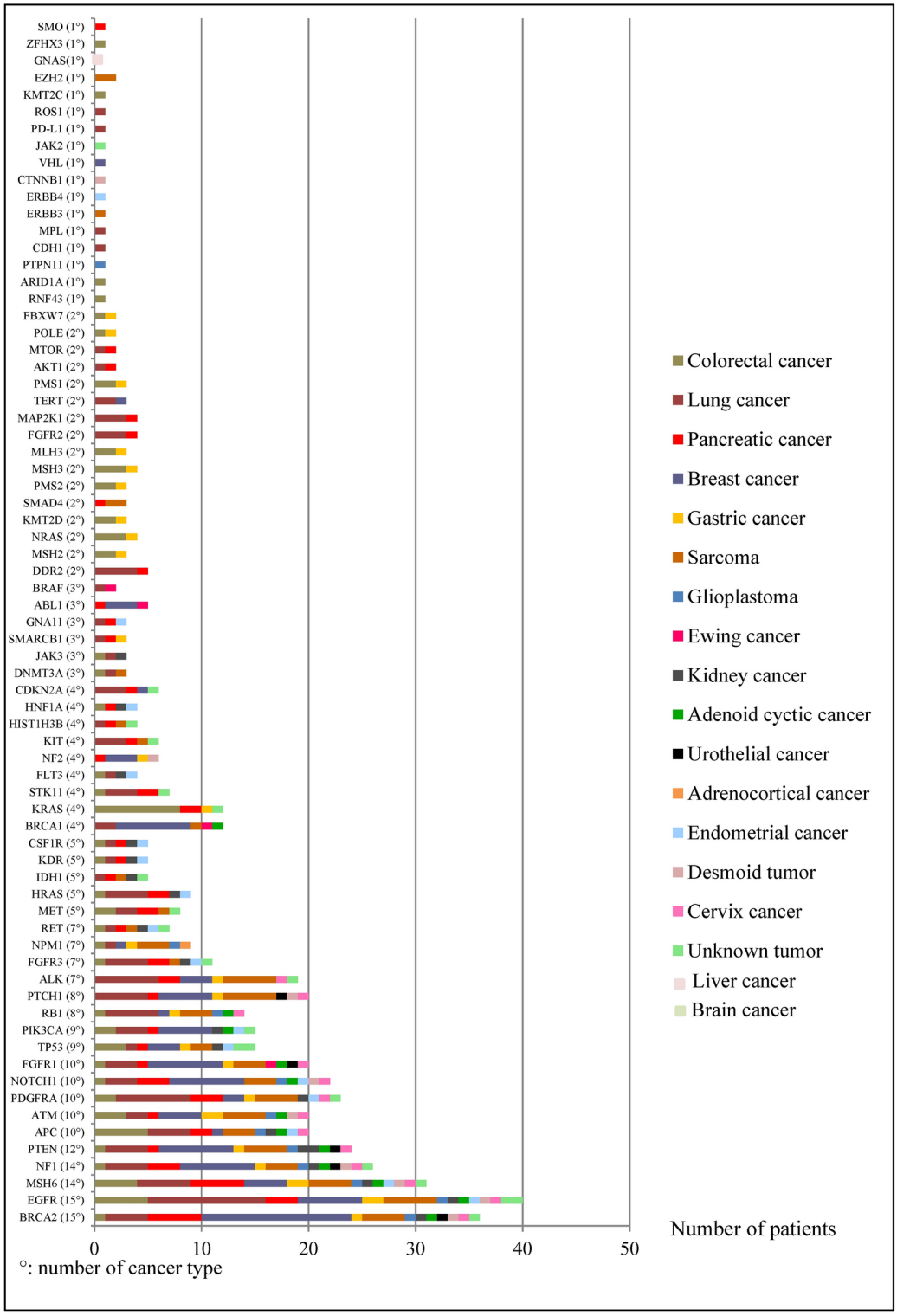

Fig. 4. Bar graph representing the number of patients with each specific gene aberration. A total of 71 genes were identified with 353 distinct aberrations.

the 76 samples with genetic alterations, 73% of them were registered with potentially actionable alterations; 64% (47 patients) had FDA approved drug and 9% (7 patients) were eligible for clinical trials (Fig. 2). Analysis of the results from these 76 tests revealed 84 distinct mutations associated with sensitivity to targeted therapies across the following investigated 19 genes: EGFR, MSH6, FGFR1, KRAS, NRAS, PDGFRA, BRCA2, ALK, JAK2, PD-L1, ROS1, TP53, IDH1, POLE, ERBB2, PIK3CA, ATM, PTCH1 and NF1.

ALK and EGFR were the most frequently altered genes associated with potential drug sensitivity, each with 13 mutations, followed by BRCA2 with 12 mutations, KRAS with 9 mutations, and PIK3CA with 6 mutations. Among the samples in our cohort, 10 samples harbored more than 7 actionable alterations. Specifically, 5 samples had 7 distinct actionable alterations, 2 samples had 12 distinct alterations, and 3 individual samples had 13, 10, and 8 actionable alterations, respectively. Interestingly, certain genetic alterations including ALK, EGFR, BRCA2, PIK3CA, KRAS presented multiple treatment options used either in combination or as monotherapy (Supplementary material, Table 2).

Mutations associated with drug resistance

To further explore the impact of tumor resistance on therapeutic strategies, an in-depth analysis of the ctDNA profiles from the 76 samples with detected mutations revealed that 15% (11 patients) of patients had mutations potentially associated with drug resistance (Fig. 2). Table 3 highlights 24 distinct mutations linked to resistance to targeted therapies across seven different genes. The study findings indicate that resistance was particularly frequent in the KRAS gene (7 mutations), followed by NF1 (5 mutations), NRAS (3 mutations), ALK (3 mutations), EGFR (2 mutations), TP53 (2 mutations), and BRAF (2 mutations).

KRAS alterations were the most common resistant mutations observed occurring in 5 patients. The KRAS mutations NM_004985.5:c.321A > T and NM_004985.5: c.35G > A were the most frequent. Our analysis also revealed strong acquired resistance in 2 patients who harbored a total of 8 resistance alterations, including 6 KRAS mutations, and 2 distinct NRAS mutations, both patients were diagnosed with colorectal cancer. Another patient affected by gastric cancer, harbored seven different resistance alterations, including four NF1 mutations, two TP53 mutations as well as one ALK mutation. Other patients harbored one or two different resistance alterations.

KRAS and NRAS alterations were predominantly resistant to Cetuximab and Panitumumab, with some variation depending on the specific mutation. Mutations in the NF1 gene were resistant to Imatinib. In the case of the ALK gene, the aberrations identified in our cohort generally showed resistance to Crizotinib, Ceritinib, and Alectinib, with slight differences related to two specific mutations: NM_004304.5: c.3593 T > C, which was sensitive to Lorlatinib, and NM_004304.5: c.3605delG, which was sensitive to Brigatinib, Crizotinib, Lorlatinib, and Erlotinib. The EGFR gene mutation NM_005228.5:c.2240_2254delTAAGAG AAGCAACAT was associated with resistance to Gefitinib and Erlotinib drugs while the NM_005228.5: c.2573 T > G mutation was sensitive to Crizotinib drug. TP53 gene alterations were resistant to IFNA2, Rituximab, Fludarabine, Cyclophosphamide, Fludarabine phosphate chlorambucil, and Lenalidomide drugs and BRAF alterations were resistant to Cetuximab, Panitumumab and Trametinib drugs (Table 4).

Discussion

With the increasing use of molecular tests, genomic ctDNA sequencing has become a valuable resource for discovering actionable gene mutations and is gaining widespread adoption in precision medicine. Here, using real world data, we 1/ evaluate the ability of detecting ctDNA in the plasma of patients in different solid cancers, 2/ identify the genes most frequently altered in various types of cancer, 3/ identify most abundant genetic alteration in well-known cancer-related genes, 4/ detect actionable mutations for targeted therapy indication, and 5/ detect the mutations associated with treatment resistance to guide therapy decision making for better clinical cancer patients management.

In the present study, significant proportions of patients (54 patients/72.97%) had test performed on plasma samples, reflecting a non-invasive approach for sequencing, which is likely more suitable for ongoing monitoring for patients where tissue samples are not available or difficult to obtain. The remaining patients had their tests performed on FFPE tissue (14 patients/18.92%) or both tissue and plasma (6 patients/8.11%), with a small subset of patients undergoing multiple tests for disease follow-up. In our analysis of the genetic profiles of the patients with tests performed on the two specimens (plasma and FFPE tissue), we found that the data generated from either liquid or tissue biopsy demonstrate similarity in terms of DNA availability, library preparation, library quality control, sequencing data output and quality, and bioinformatics analysis. However, according to genetic alteration detected, among the 6 patients who underwent both ctDNA and tissue genetic testing, only 3 patients exhibited common alterations in both their FFPE tissue and matched liquid biopsy. The remaining 3 patients had genetic alterations detected in their ctDNA samples that were not observed in their FFPE tissues. Several studies have assessed the concordance between liquid and tissue biopsy. Recent studies, including a comprehensive comparison by Bayle et al.[28], highlight the complementary roles of liquid and tissue biopsies in cancer diagnostics. Liquid biopsy offers faster turnaround times (12 vs. 28 days) and lower testing failure rates (3.9% vs. 15%), though a small subset of patients lacks detectable ctDNA alterations. When comparing actionable alterations, only 41.99% were common between liquid and tissue biopsy, with 22.94% found only in liquid biopsy and 35.07% only in tissue. These observations suggest that both biopsies remain complementary in detecting actionable mutations, and both are needed. Other studies evaluate high concordance rates between the two approaches[13]. Kang et al. reported a 93% concordance between ctDNA and tumor DNA in metastatic colorectal cancer[14]. Similarly, Corcoran's research demonstrated high concordance rates of 97–100% across multiple cancer types, reinforcing the reliability of genetic testing from liquid biopsy[15]. While our study is limited by the small number of paired samples and their restriction to advanced stages, our findings are consistent with previously published research. It is evident that concordance between tissue and liquid biopsy can be observed, but only partially, but factors such as cancer type and disease stage significantly impact the concordance rate. Several studies stress the impact of cancer type, stage, and timing on concordance rates, influenced by tumor heterogeneity and ctDNA dynamics[22–24].

Next generation sequencing approach used in this study detected a total of 353 distinct aberrations. Various genetic alterations were found and could be gathered into five categories, namely deletions, transitions,

Simple ID		2	3	4	5	35	59	62	67	68	72	78	82	85	Drugs associated with resistance
KRAS	NM_004985.5:c.436G>A p.A146T	■													Cetuximab / panitumumab
KRAS	NM_004985.5: c.321A>T p.E107D						■	■		■					Gefitinib / erlotinib -Cetuximab / panitumumab
KRAS	NM_004985.5: c.43G>A p.G15S							■		■					Cetuximab / Panitumumab
KRAS	NM_004985.5: c.37G>A p.G13S							■		■					Cetuximab / irinotecan / panitumumab
KRAS	NM_004985.5: c.35G>A p.G12D							■		■		■			Cetuximab / panitumumab / Vemurafenib
KRAS	NM_004985.5: c.48G>A p.K16K							■		■					Cetuximab / panitumumab
KRAS	c.20delTinsGC p.V7fs*27							■		■					Cetuximab / panitumumab
EGFR	NM_005228.5:c.2240_2254delTAAGAG AAGCAACAT p.L747_T751del		■												Gefitinib / erlotinib
EGFR	NM_005228.5: c.2573T>G p.L858R			■											Crizotinib
ALK	NM_004304.5: c.3605delG p.G1202fs*56					■			■		■		■		Crizotinib/ceritinib/ brigatinib/ alectinib- Crizotinib/ Lorlatinib / Erlotinib
ALK	NM_004304.5: c.3593T>C p.L1198P								■						Crizotinib / Lorlatinib / ceritinib / alectinib
ALK	NM_004304.5: c.3606A>G p.G1202G										■				Crizotinib /ceritinib / alectinib
TP53	NM_001276699.2: c.-1207_-1191delACCTGGAGGGCTGGGGG _____					■									IFNA2 / fludarabine/ cyclophosphamide/ fludarabine phosphate chlorambucil / lenalidomide / rituximab
TP53	NM_000546.6: c.1146delA p.K382fs*?					■									
NRAS	NM_002524.5: c.204A>G p.R68R						■	■		■					Cetuximab / panitumumab
NRAS	NM_002524.6: c.321T>A p.D107E							■							Cetuximab / fluoropyrimidine / panitumumab
NRAS	NM_002524: c.322G>A p.D108N									■					Cetuximab / panitumumab
BRAF	NM_004333.6:c.1897T>C p.Tyr633His													■	Cetuximab / Panitumumab / Trametinib
BRAF	NM_004333.6:c.1820C>T p.Ser607Phe													■	
NF1	NM_000267.3:c.1400C>T p.T467I				■										Imatinib
NF1	NM_000267.3: c.2033delC p.P678fs*10				■	■									Imatinib
NF1	NM_000267.3: c.655-34delA _____					■									Imatinib
NF1	NM_000267.3: c.4515-23delT _____					■									Imatinib
NF1	NM_000267.3: c.7908-8dupT _____					■									Imatinib

Table 4. Acquired resistance mutations in patients with different cancers.
24 distinct mutations associated with resistance across 7 distinct genes in plasma and tumor samples were found in our cohort

transversions, duplications and deletion/insertion. The deletions (47%) and transitions (30%) were the most commonly identified DNA alterations in our cancer population and were predominant in all cancers except for endometrial cancer, where deletions were not found. Deletions in cancer cells frequently occur at fragile, mutation-prone regions of the genome and within genes that encode tumor suppressors[29]. Our data are consistent with Fitch's observations. Transitions (purine-to-purine or pyrimidine-to-pyrimidine changes) are more frequent than transversions (purine-to-pyrimidine or pyrimidine-to-purine changes). Transitions are more likely to conserve the important biochemical properties of the original amino acid, whereas transversions often result in a radical amino acid change. Consequently, transversions tend to be more detrimental than

transitions[31]. In cancer genetic counseling, greater focus should be placed on transversion alterations to identify potential driver mutations. The underlying reasons for the imbalance between transition and transversion biases remain largely unknown. Several hypotheses have been proposed, including the role of natural selection. In the context of tumorigenesis, the dynamic interplay between natural selection, the fidelity of polymerase function, and driver mutation pressure may provide a plausible explanation. Gene duplications are commonly observed in human cancers, with many of the resulting products being pathogenic and serving as key drivers of tumorigenesis and cancer progression. In some cancer subtypes, the duplication and fusion of genes are critical for the initiation of tumor formation, and in certain cases, they have the potential to transform normal cells[30].

The innovation of NGS approach allows the detection of all range of genetic alterations, if the suitable sequencing library is performed, such as pair-end library, mate pair-library or the using of long read sequencing. Unfortunately, in our study and in most routine clinical molecular biology practice, the test uses the targeted pair-end library with short read sequencing, which limits the analysis to only to small scale genetic alterations but present the advantage to be less expensive. Recently, methods based on molecular testing using ctDNA focus mainly on the analysis of alterations in of well-known cancer related genes. Generally, the aberrations in these genes could be identified using several technologies, such as polymerase chain reaction (PCR) or next generation sequencing approaches. PCR technologies reveal only the presence of known mutations, known as hotspot mutations, while the NGS approaches examine the whole sequence of gene of interest and can detect any known or unknown genetic variants[16]. In the present study, NGS allowed us to detect various genetic alterations that could be present in the gene sequence. We found that 81% (60 patients) of our patients presented with at least two mutations, 8% of patients (6 patients) harbored one mutation and 11% of patients (8 patients) had no detected mutation. It is fortunate but relatively rare to encounter a single, actionable mutation in a patient. Most of our patients were found with several mutations. The complexity of tumorigenesis specifically in patients with metastasis cancer, produce often more genetic alterations, which make managing these patients a challenging task. A basic scientific investigation focuses on a single alteration that appears to be a pathogenic and cancer driver, for which a targeted treatment is available. As previously described and published, the reductionist paradigm has been revolutionized and shifted to a systems approach in research[17]. Appropriate bioinformatics tools are needed in clinical practice to understand the combinatory action of all alterations found in a patient, in order to suggest the treatment that could probably give high probability of success. Unfortunately, clinical practice still lags and suffers from this limitation.

Of note is that we found patients at advanced stages who had high quantities of circulating DNA in their plasma, and for which the sequencing library was successful, with no identified mutations, a finding which might due to several factors, including low tumor burden, poor vascularization of tumor, high rates of cell necrosis and apoptosis, low ctDNA release by some tumor subtypes, or the absence of detectable tumor traces at the time of blood sampling. CtDNA concentration may decrease after surgical resection or during treatment, as noted by several researchers[2,6]. Additionally, the total volume of plasma derived from the blood can present a technical limitation in detecting genetic alterations, especially when the amount of released tumor DNA is very limited compared to the normal circulating DNA in the plasma. This can make detecting these tumor variants challenging if the variant fraction is very low compared to the wild-type allele[18,20]. Somatic mutations are often less detectable in ctDNA due to tumor heterogeneity, limited ctDNA volume, and detection method limitations, particularly for low-frequency mutations[25,26]. Tumors also vary in DNA shedding, with early-stage cancers releasing less ctDNA (25). Conversely, germline mutations are strongly associated with hereditary cancer syndromes, often leading to significant cfDNA release due to early-onset, aggressive tumor characteristics, and their ubiquitous presence throughout the body[27]. The absence of ctDNA does not guarantee the absence of actionable alterations, emphasizing the early stage of ctDNA integration into clinical practice. Opening the door to more molecular biology tools and protocols is essential for effective implementation, which could significantly accelerate the adoption of precision medicine[21]. Recent recommendations from European Society for Medical Oncology (ESMO) suggest performing tumor NGS in clinical research centers. In this context, we believe that, compared to clinical laboratories, these research centers offer greater flexibility in techniques and protocols, which could benefit patients in whom no detectable circulating DNA is identified.

Driver mutations play a critical role in tumor growth and survival. The goal of precision medicine is to provide treatments specifically targeting these pathogenic mutations. A recent review by experts from the ESMO suggests that ctDNA assays should be considered in clinical practice when tissue biopsy is either not feasible or unsuitable for the patient, and when rapid results are required[19]. Our findings align with recent recommendations to perform tumor NGS to detect tumor alterations in patients with metastatic cancers, where access to matched therapies is available. In our study of the 74 patients enrolled, 88% (65 patients) of patients were eligible for treatment guidance using liquid biopsy. Specifically, 64% (47 patients) of patients could potentially benefit from FDA-approved drugs, 9% (7 patients) could qualify for clinical trials involving experimental drugs, and 15% (11 patients) needed to avoid drugs associated with tumor resistance. This study is the first to show that a significant number of patients can benefit from personalized medicine through a simple, non-invasive genetic test across various cancer types. The ctDNA analysis in this study identified actionable mutations in 73% of samples with alterations, revealing 84 mutations linked to therapeutic sensitivity across 19 genes, with EGFR, ALK, BRCA2, KRAS, and PIK3CA being the most frequently altered. Additionally, 15% of patients exhibited resistance-associated mutations, with 24 distinct mutations across 7 genes, notably in KRAS, NF1, NRAS, ALK, EGFR, TP53, and BRAF. Monitoring and early detection of these resistance mechanisms can offer snapshots of resistant mutations at any stage of the disease, aiding physicians in initiating new therapies to limit or potentially eradicate tumor growth. It would be interesting to follow up with patients receiving personalized medicine and compare their outcomes to those who do not undergo genetic testing in future studies. Our study clearly demonstrates the benefits of using liquid biopsy, and we hope to see additional recommendations for the systematic incorporating

of such noninvasive tests into comprehensive patient management, irrespective of cancer type, tumor stage, or treatment availability.

Our study demonstrates that circulating tumor DNA (ctDNA) can serve as an effective non-invasive assay for managing patients regardless of cancer type. It allows for the detection of genetic alterations at frequencies comparable to those observed in tissue sequencing, identifies mutations associated with resistance, and guides therapeutic decisions. Our findings suggest that, in the area of precision medicine, molecular testing using next-generation sequencing (NGS) offers valuable insights and may be crucial for patient management, supporting the usefulness of ctDNA profiling in identifying the pathogenic mutations of patients with cancer and providing clinically relevant insights into tumor heterogeneity and therapeutic resistance.

Data availability

The data that support the findings of this study are available on request from the corresponding author [h.mansour@ump.ac.ma]. The data are not publicly available due to restrictions [e.g. they contain information that could compromise the privacy of participants].

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Author contributions

HM planned, set up the experiment workflow and supervised the work, NA & AO supervised the patients recruitment and follow up. OM extracted and analyzed the data. OM & HM wrote the paper. RI, AO, CN and NA reviewed the data and edited the paper. All authors contributed to the article and approved the submitted version.

Competing interests

The authors declare no competing interests.

Additional information

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