

Custom multi-tumor next-generation sequencing panel for routine molecular diagnosis of solid tumors: Validation and results from three-year clinical use

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Abstract. Molecular testing is extremely important in cancer care, starting as early as at diagnosis. In order to address the challenge of providing reliable results within the timeframe adapted to patient management and suitable to guide clinical decisions, a capture-based next-generation sequencing (NGS) panel focusing on ten genes known to harbor genetic variations which may be targeted by approved drugs in patients with cancer was designed and validated. Very favorable analytical performances were obtained for both solid and liquid biopsies. For solid biopsies, a low read depth (80X per nucleotide) led to the genotype detection accuracy of 100%. The read of raw data for liquid biopsies resulted in the 91.19% result concordance between paired solid and liquid samples. The present method met all the requirements for the ISO15189 certification. During our three-year experience of routinely using this panel, almost 2,300 samples from lung and colorectal cancers, melanomas and gastrointestinal stromal tumors have been analyzed. It was found that our panel detected slightly more gain-of-function variants than described in the literature. Surprisingly, loss-of-function variants were also detected in certain of the analyzed genes. Finally, liquid biopsy data revealed statistically different mutated allele frequencies between tumor types, but also between mutated genes and variants themselves. In conclusion, the use of our capture-based NGS panel

is perfectly adapted to perform relevant molecular diagnosis in a time frame compatible with patient care.

Introduction

For years, the pathological evaluation of tumor tissue has been essential for cancer diagnosis and care. The field of pathology has been becoming more and more complex, requiring the pathologists to integrate new techniques and develop new skills. With the advent of targeted therapies, this has included the use of molecular biology techniques. The application of molecular biology to cancer diagnostics started 15-20 years ago with the fluorescence *in situ* hybridization analysis of the *ERBB2* gene in breast cancer (1), followed by sequence analysis of *EGFR* and *KRAS* in lung and colon cancers (2). At present, an increasing number of genes are analyzed, irreversibly transforming 'classic' pathology into modern molecular pathology. However, one of the major challenges of the molecular diagnosis of patients with cancer is to be able to fit the suitable target analysis into the right (from a clinical standpoint) timing. The latter should allow the diagnosticians to obtain objective (uninfluenced by the first-line treatment) molecular results. Concurrently, the results must be provided early enough to guide treatment decisions integrating precision medicine solutions in time to obtain favorable outcomes in metastatic patients.

Recent years have witnessed tremendous technological development which has revolutionized cancer diagnostics. In particular, the development of next-generation sequencing (NGS) has significantly increased analytical capacities, which has enabled laboratories to analyze increasing numbers of targets and indicators. Molecular analyses have become standard for diagnosis of several solid cancers in Europe. These analyses cover common and different target genes that could be mutated in tumors: *ALK* (3), *BRAF* (4), *EGFR* (5), *ERBB2* (6), *KRAS* (7), and *MET* (8) in lung cancers, *BRAF* (9), *KRAS* (10), and *NRAS* (11) in colorectal tumors, *BRAF* (12), *KIT* (13),

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and *NRAS* (14) in melanoma, as well as *KIT* and *PDGFRA* in gastrointestinal stromal tumors (GISTs) (15). These genes are known to be mutated at hotspots, but the analytical performance of the NGS technology allows us to analyze larger gene regions, or even whole coding parts of these genes, which may result in important clinical implications.

In the present study, an NGS custom panel which was designed and validated for molecular diagnosis of lung cancer, colorectal cancer, melanoma and GISTs based on the analysis of solid and liquid biopsies was presented. This NGS custom panel has been used successfully in our clinical practice for three years.

Materials and methods

Patients and samples. The present study was conducted on solid tumor samples from 2,289 patients diagnosed at the Georges Francois-Leclerc Cancer Center (CGFL; Dijon, France) between January 2017 and December 2019. All tumor samples were sent to our lab for analyses as part of the routine clinical diagnosis procedure. They included 1,299 lung cancer samples, 790 colorectal cancer samples, 158 melanomas and 42 GISTs. Formalin-fixed paraffin-embedded tumor blocks were obtained from different pathology laboratories. Additionally, blood samples (liquid biopsies) were collected into special 10-ml cell free DNA collection tubes whenever possible for plasma DNA extraction and analysis. The present study on patient samples was conducted in accordance with the Declaration of Helsinki and was approved (approval no. 00010311) by the Ethics Committee of the Georges-François Leclerc Cancer Center (Dijon, France) and by the Consultative Committee of Burgundy (Dijon, France) for the Protection of Persons Participating in Biomedical Research (Comité Consultatif de Protection des Personnes en Recherche Biomédicale de Bourgogne). Written informed consent was provided by all patients.

DNA extraction. Tumor slides prepared from formalin-fixed paraffin-embedded tumor specimens were analyzed by a pathologist to determine tumor cell content. Only specimens containing more than 5% of tumor cells were used for DNA extraction. DNA was extracted from five 8- μ m-thick tumor slides per patient using the Maxwell-16 formalin-fixed paraffin-embedded Plus LEV DNA purification kit (Promega Corporation) according to the manufacturer's protocol and quantified using a fluorimetric assay on a Qubit device 4 (Life Technologies; Thermo Fisher Scientific, Inc.).

In order to separate blood plasma, liquid biopsies were centrifuged at 1,150 x g for 6 min at room temperature. Then, the supernatant was transferred into a new tube and centrifuged at 12,000 x g for 15 min at 4°C. Next, DNA was extracted from ~2 to 4 ml of plasma using the QIAamp circulating nucleic acid kit (Qiagen GmbH) according to the manufacturer's protocol.

Panel design and library preparation. A panel focusing on coding regions (-5/+5 nucleotides in flanking introns except for *MET* exon 14 flanking introns for which -80/+80 nucleotides were analyzed) of ten genes related to approved treatments for lung and colorectal cancers, melanomas and GISTs was

developed: *ALK*, *BRAF*, *EGFR*, *ERBB2*, *KIT*, *KRAS*, *MAP2K1*, *MET*, *NRAS*, and *PDGFRA* (Table I). Coding regions of the *TP53* gene (detectable in liquid biopsies) as well as five regions containing single nucleotide polymorphisms (SNPs) were also included to control for sample cross-contamination (Table II) (16). The panel design was patented under the number WO2019/197541.

The panel performances were evaluated on 15 criteria required to obtain the ISO15189 accreditation (ISO15189:2012). The latter is necessary to obtain the accreditation by the French Accreditation Committee (COFRAC), obligatory for routine molecular diagnosis laboratories in France.

For library preparation, 400 ng of DNA from solid tumors were fragmented with a Covaris LE220-plus device (Covaris, Inc.) to obtain fragments ~300 bp-long. For liquid biopsies, 30 μ l of DNA were directly engaged in the process, without shearing. Then, libraries were prepared with the SureSelect^{XT} (cat. no. G9642B) technology (Agilent Technologies, Inc.) following the manufacturer's instructions. Paired-end (2x111 bases) sequencing was performed on a MiSeq device (Illumina, Inc.) twice a week. For solid samples, all hotspot mutations were confirmed by allelic discrimination, fragment analysis or Sanger sequencing for identity checking.

Bioinformatic analysis. Reads in FASTQ format were aligned to the reference human genome GRCh37 using the Burrows-Wheeler aligner (BWA v.0.7.15) (17). Local realignment was performed using the Genome Analysis Toolkit (GATK v.3.6) (18-20). Duplicate reads were removed using Picard v.2.5 (21). Outlyzer (v1.0) (22) was used to identify variants. These variants were annotated using Annovar (Annovar2016Feb01) (23) and SnpEff (v4.3i) (24). Quality controls were performed using fastQC (v0.11.8) (25), Samtools (v1.9) (26), and Qualimap (v2.2.1) (27) information through multiQC (v1.7) software (28).

Variants with a frequency superior to 1% in the general population were filtered and did not appear in results tables analyzed by the biologists.

Statistical analysis. Analysis was performed with GraphPad Prism version 8.3.0 (GraphPad Software, Inc.). No statistical analyses were performed for Figs. 1A and 4 and S1B. Concerning Fig. 4C and D, non-parametric Kruskal-Wallis test was used followed by Dunn's test. For Fig. 4C, n=287 in the lung subset, n=78 in the colon subset and n=18 in the melanoma subset. For Fig. 4D, n=81 for EGFR, n=50 for RAS/BRAF/MAP2K1 group, and n=81 for TP53 subset. For Fig. 4E, non-parametric Mann-Whitney U-test was used; the EGFR-sensitive mutation subset had 85 cases and resistance mutation sets had 27 cases. P<0.05 was considered to indicate a statistically significant difference.

Results

Panel description and performances. An NGS-based panel for routine molecular diagnosis of four tumor types was developed: Lung carcinoma, colon carcinoma, melanoma and GIST. Our panel includes the coding regions of ten genes related to approved treatments for these four tumor types (Table I). It also includes the coding regions of the *TP53* gene to detect tumor

Table I. Genes included in our next-generation sequencing panel and clinical impact of their activating variants.

Gene	Tumor type	Associated treatment or prognostic effect	Impact
<i>ALK</i>	Lung cancer	ALK TKI	Sensitivity of resistance to ALK inhibitor
<i>BRAF</i>	Colon cancer	No therapeutics	MSI Testing
	Lung cancer	BRAF inhibitor	Sensitivity only for the p.(Val600Glu) variant
	Melanoma	BRAF inhibitor	Sensitivity only for p.(Val600X) variants
<i>EGFR</i>	Lung cancer	EGFR TKI	Sensitivity or resistance (secondary mutations, insertions exon 20)
<i>ERBB2</i>	Lung cancer	Mobocertinib	Sensitivity
<i>KIT</i>	GIST	Imatinib	Sensitivity or resistance (secondary mutations)
	Melanoma	Imatinib	Sensitivity
<i>KRAS</i>	Colon cancer	EGFR inhibitor	Resistance
	Lung cancer	Sotorasib	Sensitivity only for the p.(Gly12Cys) variant
<i>MAP2K1</i>	Melanoma	BRAF inhibitor	Resistance
		MEK inhibitor	Sensitivity or resistance
<i>MET</i>	Lung cancer	MET TKI	Sensitivity
<i>NRAS</i>	Colon cancer	EGFR inhibitor	Resistance
	Melanoma	Prognosis	/
<i>PDGFRA</i>	GIST	Imatinib	Sensitivity or resistance (secondary mutations)

GIST, gastrointestinal stromal tumor; MSI, microsatellite instability; TKI, tyrosine kinase inhibitor(s).

Table II. List of five regions containing SNPs integrated in our panel for detecting putative cross-contamination between samples.

Gene	Amino acid variation	Nucleotide variation	European population frequency (%) ^a	African population frequency (%) ^a
<i>EGFR</i>	Q787Q	c.2361G/A	40.59/59.41	59.18/40.82
<i>EIF1AY</i>	Gene present only on the Y chromosome			
<i>KDR</i>	Q472H	c.1416A/T	76.03/23.97	88.19/11.81
<i>SLC28A1</i>	V189I	c.565G/A	64.85/35.15	78.62/21.38
<i>TP53</i>	R72P	c.215G/C	26.34/73.66	68.54/31.46

^aData retrieved from dbSNP [adapted from Ref (50)]. SNP, single nucleotide polymorphism.

material in liquid biopsies as well as five regions containing SNPs to control for sample cross-contamination (29).

In order to comply with the French regulations on routine molecular diagnosis laboratories, our panel was assessed for 15 items according to the ISO15189 standards. Our method met all the requirements for the ISO15189 accreditation (Table III). Repeatability was calculated using five replicates of the Horizon HD728 internal control (AmpliTech Sarl) with a specific focus on two-point variants: G719S in the *EGFR* gene and G12A in *KRAS*. The *EGFR* G719S mutation had a mean detection frequency of 17.6±1.8% (expected frequency, 16.7%) with a variation coefficient of 10.2%, while *KRAS* G12A was detected with a mean frequency of 3.9±0.8% (expected frequency, 5%) with a variation coefficient of 20.2%. Intermediate fidelity was assessed on the same variants by analyzing 20 replicates. *EGFR* G719S was detected with a mean frequency of 17.2±0.9% (expected frequency, 16.7%) with a variation coefficient of 5.1% and

KRAS G12A had a mean detection frequency of 4.1±0.6% (expected frequency, 5%) with a variation coefficient of 13.5%. Using the same 20 replicates, the rightness of our panel was determined to obtain a bias of 3% for the *EGFR* G719S mutation and -18% for the *KRAS* G12A mutation. Subsequently, to calculate the accuracy of our technique, nine samples received from the 2017 European Molecular Genetics Quality Network (EMQN) campaign were used. The expected genotype was detected in all samples, thus showing a 100% accuracy of the technique for genotype detection and an 89% accuracy for the alternative allelic frequency (Fig. S1A). The alternative allelic frequency of eight variants from the Horizon HD728 control on 231 consecutive runs was also examined (Fig. S1B). Except for the *BRAF* V600E mutation for which the alternative allelic frequency was slightly overestimated, all alternative allelic frequencies were equal to expected values. Based on the analysis of the same 2017 EMQN campaign samples, a sensitivity and specificity of 100% was obtained. These values were

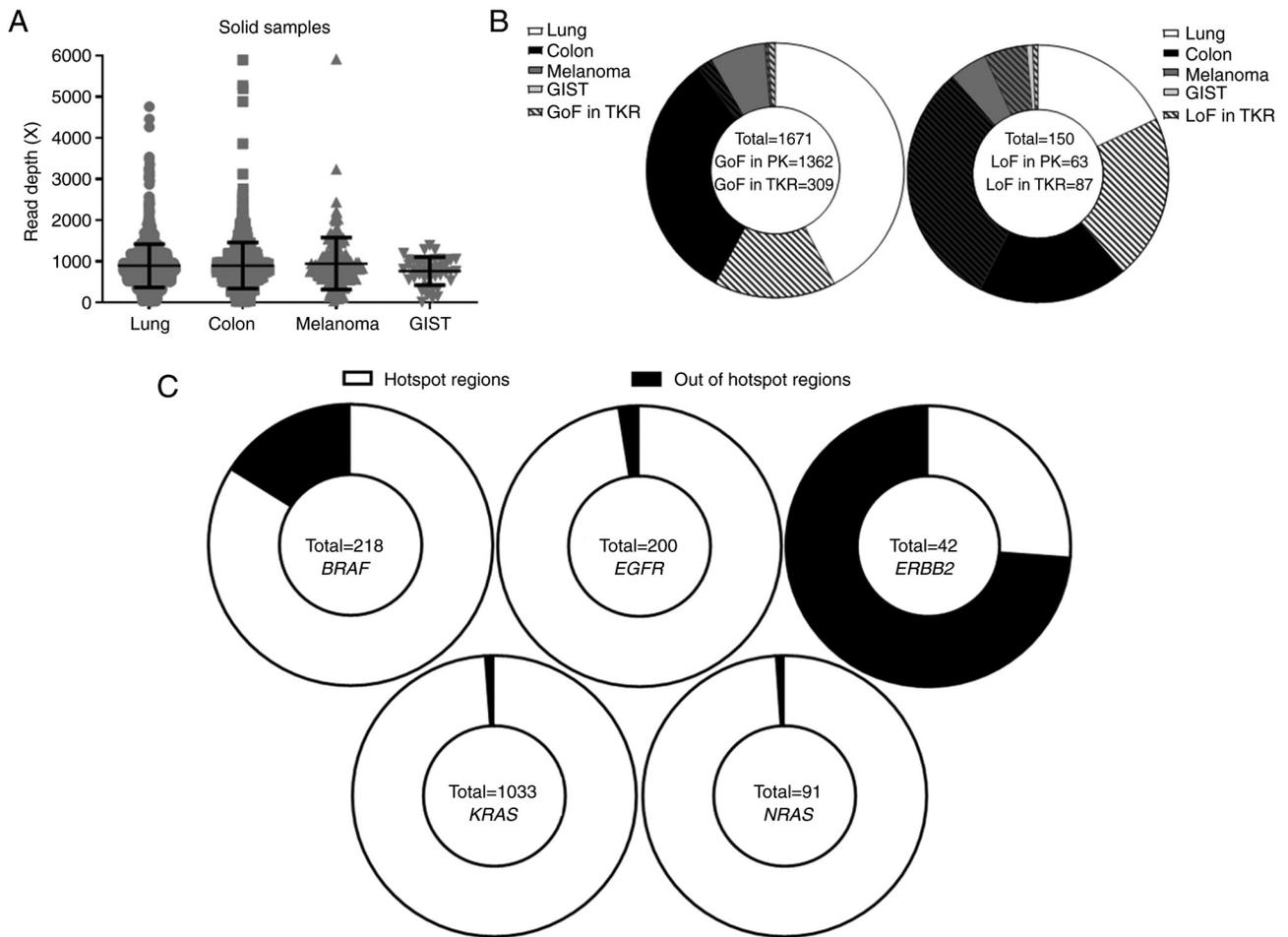


Figure 1. Landscape of variants detected in tumor samples from four tumor types (lung carcinoma, colon carcinoma, melanoma and GIST by our custom next-generation sequencing panel. (A) Read depth coverage obtained for solid biopsies by tumor type. (B) Distribution of GoF variants (left) and LoF variants (right) between genes encoding PK and those encoding TKR, by tumor type. (C) Distribution of GoF mutations in *BRAF*, *EGFR*, *ERBB2*, *KRAS* and *NRAS* between hotspot and non-hotspot regions. GIST, gastrointestinal stromal tumor; GoF, gain-of-function; LoF, loss-of-function; PK, protein kinase; TKR, tyrosine kinase receptor.

confirmed by benchmarking using 231 runs with the Horizon HD728 control. Additionally, during the three-year period analyzed, the presence of all variants of interest ($n=1,505$) found in *ALK* ($n=1$), *BRAF* ($n=51$), *EGFR* ($n=166$), *ERBB2* ($n=11$), *KIT* ($n=30$), *KRAS* ($n=1018$), *MET* ($n=34$), *NRAS* ($n=90$), and *PDGFRA* ($n=4$) was confirmed by other targeted technical approaches. Finally, our very low background ($<1\%$) enabled us to set the detection limit at 1% (particularly for hotspot regions). For liquid biopsies, the systematic visualization of raw data (bam files) decreased the detection limit below 0.5%.

Mutational landscape in tumor samples. From January 2017 to December 2019, 2,289 solid tumor samples from lung ($n=1,299$), colon/rectum ($n=790$), melanoma ($n=158$), and GIST ($n=42$) were sequenced with our custom NGS panel. The median read depth obtained was 828.52X [lung cancer, 832.94X; colorectal cancer, 818.16X; melanoma, 842.36X; and GIST, 800.10X (Fig. 1A)]. A total of 1,180 different variations that were classified as benign ($n=67$), unknown ($n=797$), gain-of-function (GoF; $n=196$) or loss-of-function (LoF; $n=110$) were observed for each gene (Tables IV and SI). In total, 1,671 of the detected variants were GoF variants. They were distributed between protein kinase-coding genes (81.5% of variants for all tumor

types together, including 42.43% in lung cancers, 32.14% in colon cancers, 6.88% in melanomas and 0.05% in GISTs) and those encoding tyrosine kinase receptors (18.5% for all the four tumor types: 15.26% in lung cancers, 1.98% in colon cancers, 0.36% in melanomas and 0.9% in GISTs; Figs. 1B left and S2). LoF variants in protein kinase-coding genes (42% for all tumors types: 18% in lung cancers, 18.67% in colon cancers, 4.66% in melanomas, and 0.67% in GISTs) were also observed, mostly in the *BRAF* gene which harbored 89.23% of all LoF variants in protein kinase-coding genes. Surprisingly, 58% of LoF variants occurred in genes encoding the tyrosine kinase receptor (20.67, 31.33, 5.33 and 0.67% for lung, colon, melanoma and GIST, respectively; Figs. 1B right and S2). The location, number, and tissue distribution of GoF and LoF variants for each gene are presented in Fig. S3. Notably, for *BRAF*, *EGFR*, *ERBB2*, *KRAS* and *NRAS* genes, certain of the detected GoF variants were different from known hotspot variants (Fig. 1C). *BRAF* and *ERBB2* genes were particularly affected. *BRAF* hotspot is located at codon 600 where activating variants for 183 (83.94%) patients were detected. A total of 35 patients (16.06%) were carriers of activating variants in the *BRAF* gene, located at codons 257, 464, 469, 483, 485, 486, 499, 597, 599, 601, and 731 (Table SI). Concerning

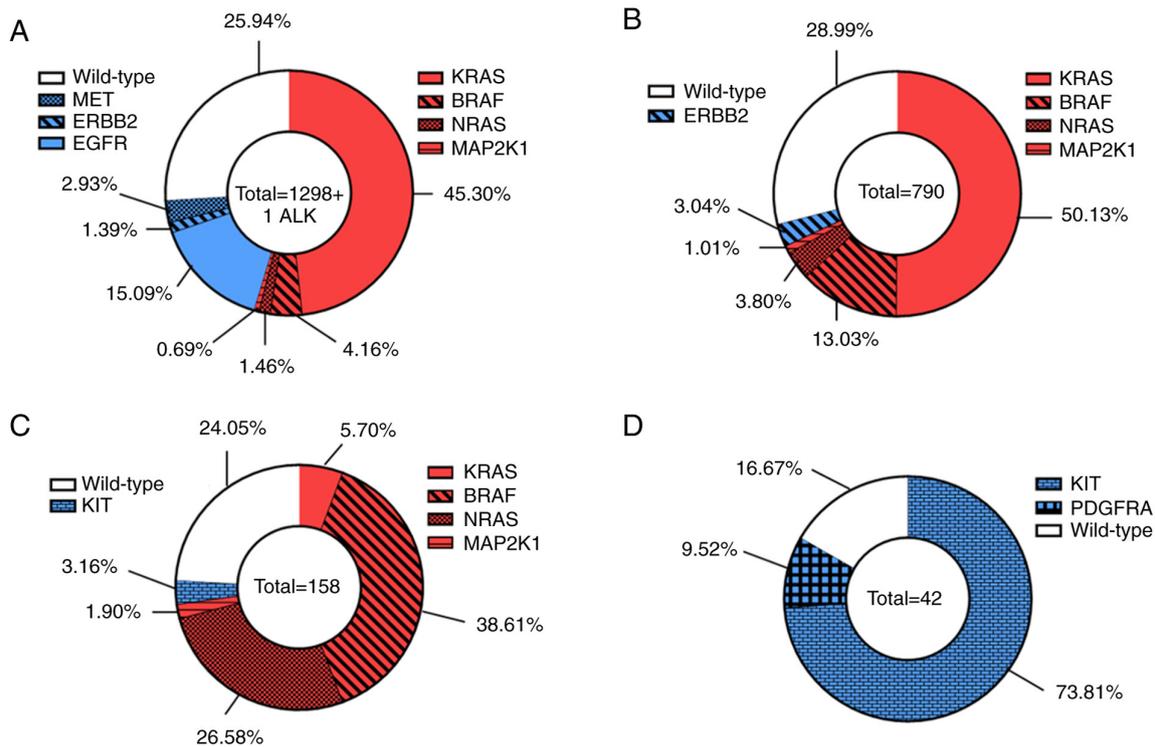


Figure 2. Distribution of activating variants between different genes of our custom next-generation sequencing panel. (A) Lung cancers, (B) colorectal cancers, (C) melanomas, and (D) gastrointestinal stromal tumors. Wild-type samples appear in white, protein kinase-coding genes in red and those encoding tyrosine kinase receptors in blue.

ERBB2, the analyzed hotspot region was exon 20, known in particular for frequent insertions in lung adenocarcinoma. A total of 11 (26.19%) insertions were detected. As expected, all of them were found in lung adenocarcinomas. Surprisingly, 31 (73.81%) point variants out of exon 20 (except for one-point mutation) were detected, concerning mainly colon adenocarcinomas (n=24; 77.42%; Fig. S2). For *EGFR*, *KRAS*, and *NRAS* genes, GoF variants were mostly in hotspot regions (Fig. 1C).

Distribution of activating variants by tumor type. It was identified that 51.61% of the 1,299 lung adenocarcinomas harbored activating variants in the RAS/RAF/MEK pathway, 19.41% had a GoF mutation in a tyrosine-kinase domain receptor, and 25.94% were wild-type. The most frequently altered gene was *KRAS* (45.3%), followed by *EGFR* (15.09%), *BRAF* (4.16%), *MET* (2.93%), *NRAS* (1.46%), *ERBB2* (1.39%), *MAP2K1* (0.69%), and *ALK* (only one case; Fig. 2A). Out of the 790 analyzed colorectal tumors, 67.97% harbored activating variants in the RAS/RAF/MEK pathway, 3.04% had a GoF mutation in a tyrosine-kinase domain receptor and 28.99% were wild-type. The most frequently altered gene was *KRAS* (50.13%) followed by *BRAF* (13.03%), *NRAS* (3.80%), *ERBB2* (3.04%) and *MAP2K1* (1.01%; Fig. 2B). As regards melanoma, 72.79% harbored activating mutations in the RAS/RAF/MEK pathway, 3.16% had a GoF mutation in a tyrosine-kinase domain receptor, and 24.05% were wild-type. The most frequently altered gene was *BRAF* (38.61%), followed by *NRAS* (26.58%), *KRAS* (5.7%), *KIT* (3.16%), and *MAP2K1* (1.9%; Fig. 2C). Finally, none of the GISTs were mutated in the RAS/RAF/MEK pathway, while GoF variants in a tyrosine-kinase domain receptor were detected in 83.33% of these

tumors and 16.67% of tumors were wild-type. Only two genes were mutated in GISTs, the majority of tumors carrying mutations in *KIT* (73.81% mutant tumors), followed by *PDGFRA* (only 9.52%; Fig. 2D).

Exon distribution of activating variants in the different genes. Since GoF variants are often located in hotspot regions, the exon distribution for all the genes on the panel was examined, except for *ALK* which had only one activating variant (Table SII). The *BRAF* gene harbored 87.15% (n=190) of activating variants in exon 15, followed by 11.47% (n=25) in exon 11, and 1.38% in exons 6, 12 and 18 (one variant in each exon; Fig. 3A). For the *EGFR* gene, 1.49% of activating variants were in exons 6 and 7 (n=1 for each), 4.46% (n=9) in exon 18, 41.58% (n=84) in exon 19, 17.82% (n=36) in exon 20 and 34.65% (n=70) in exon 21 (Fig. 3B). Activating variants were distributed among seven different exons in the *ERBB2* gene, with 14.29% (n=6) in exon 8, 21.43% (n=9) in exon 12, 21.43% (n=9) in exons 17 (n=5), 18 (n=1), and 19 (n=3), 28.56% (n=12) in exon 20 and 14.29% (n=6) in exon 21 (Fig. 3C). The 39 GoF variants in the *KIT* gene were located in five exons, with 5.13% (n=2) in exons 8 and 9, 87.18% (n=34) in exon 11, and 7.69% (n=3) in exons 14 (n=1) and 17 (n=2) (Fig. 3D). The most often mutated gene, *KRAS*, had 89.84% (n=928) of GoF variants in exon 2, 6% in exon 3 (n=62) and 4.16% (n=43) in exon 4 (Fig. 3E). The 20 GoF variants of the *MAP2K1* gene were mostly in exon 2 (65%, n=13), followed by 25% (n=5) in exon 3, and 10% (n=2) in exon 6 (Fig. 3F). Only three exons harbored activating variants in the *MET* gene, with 92.50% (n=37) located in exon 14 under the form of splicing variants, and 7.50% in exons 16 (n=2) and 17 (n=1; Fig. 3G). *NRAS*

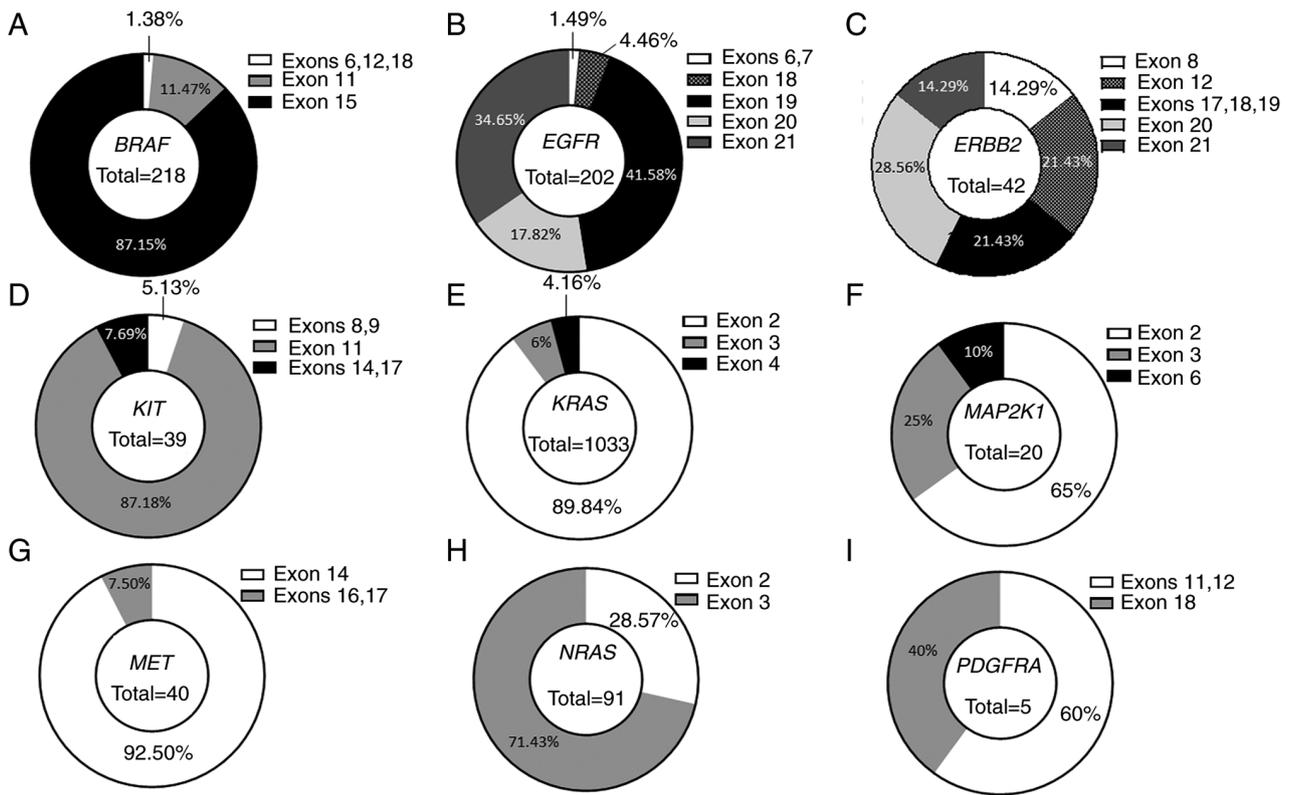


Figure 3. Distribution of activating variants between exons of genes analyzed with our custom next-generation sequencing panel. Percentages of activating variants detected in (A) *BRAF*, (B) *EGFR*, (C) *ERBB2*, (D) *KIT*, (E) *KRAS*, (F) *MAP2K1*, (G) *MET*, (H) *NRAS* and (I) *PDGFRA* are indicated for each exon.

harbored 28.57% (n=26) variants in exon 2 and 71.43% (n=65) in exon 3 (Fig. 3H). The least mutated gene, *PDGFRA*, had 60% of variants in exons 11 (n=1) and 12 (n=3), and 40% (n=2) in exon 18 (Fig. 3I).

Use of the panel for analyzing liquid biopsies. In recent years, liquid biopsies have been routinely used in clinical practice, particularly for monitoring patients with *EGFR*-mutant lung cancer treated with an *EGFR* tyrosine kinase inhibitor. They are also used for primary diagnosis of lung cancer and monitoring of disease progression in patients with other cancer types. In order to keep fluidity in result release, three years ago the analyses of liquid biopsy samples were integrated in the same wet lab flow as those of solid samples. During these three years, 454 liquid samples from 340 patients were analyzed, obtaining a mean coverage of 592.8X for lung cancer samples (n=356), 600.8X for colorectal cancer samples (n=81) and 601.1X for melanoma samples (n=17; Fig. 4A). Hotspot or LoF *TP53* variants were detected in 180 out of 340 patients. Among the 160 patients in whom no variants were found, 27 had tumors with no alterations in the analyzed genes, 20 had mutant tumors and 113 had tumors of unknown genotype. Paired solid and liquid biopsy material was available for 227 patients with lung cancer, colon cancer, or melanoma (no liquid biopsies available for GIST patients). Concordant results were found for the biopsy pairs for as numerous as 91.19% of these patients, with 79.30% of them harboring gene variants which were detected in both tumor DNA and circulating tumor DNA (ctDNA) and 11.89% having wild-type tumors and no variants detected in liquid biopsies. Only 8.81% of patients had no

variants detectable in ctDNA, whereas they had mutant tumors (Fig. 4B, left panel). When focus was addressed on each cancer type, it was observed that liquid biopsies from melanoma were the most positive, followed by colon and lung cancer. Mutation in ctDNA was not detected in 2.33% of patients with colon cancer and in 3.96% of patients with lung cancer (Fig. 4B, right panel). Finally, between liquid biopsies and solid tumors, a concordance was obtained in 100% of cases with melanomas, 97.67% with colon cancers and 96.04% with lung cancer.

For further analysis of liquid biopsy samples with ctDNA variants, mutant allele frequency of these variants was compared between the different tumor types (lung, colon, and melanoma). Similar median mutant allele frequency values were found for lung cancer samples and melanomas (4.74 and 4.935%, respectively), whereas they were higher (11.53%) for colon tumors (Fig. 4C). Next, focus was addressed on lung cancer samples which were the most numerous. It appeared that the mutant allele frequencies were significantly different between the three subsets (P=0.0042). For the *EGFR* and *TP53* genes, mutant allele frequencies were not significantly different (6.778 and 9.113%, respectively; P=0.6255). Mutant allele frequencies in *EGFR* and *RAS/BRAF/MAPK1* genes were almost significantly different (6.778 and 2.445%, P=0.0634). This difference was significant between *RAS/BRAF/MAPK1* and *TP53* genes (2.445 and 9.113%; P=0.0033) (Fig. 4D). Finally, comparing mutant allele frequencies of primary *EGFR* variants (driver variants) with those of new variants appearing after progression under treatment (resistance variants) it was identified that mutated allele frequencies of driver variants were significantly higher than those of resistance variants (P=0.0035; Fig. 4E).

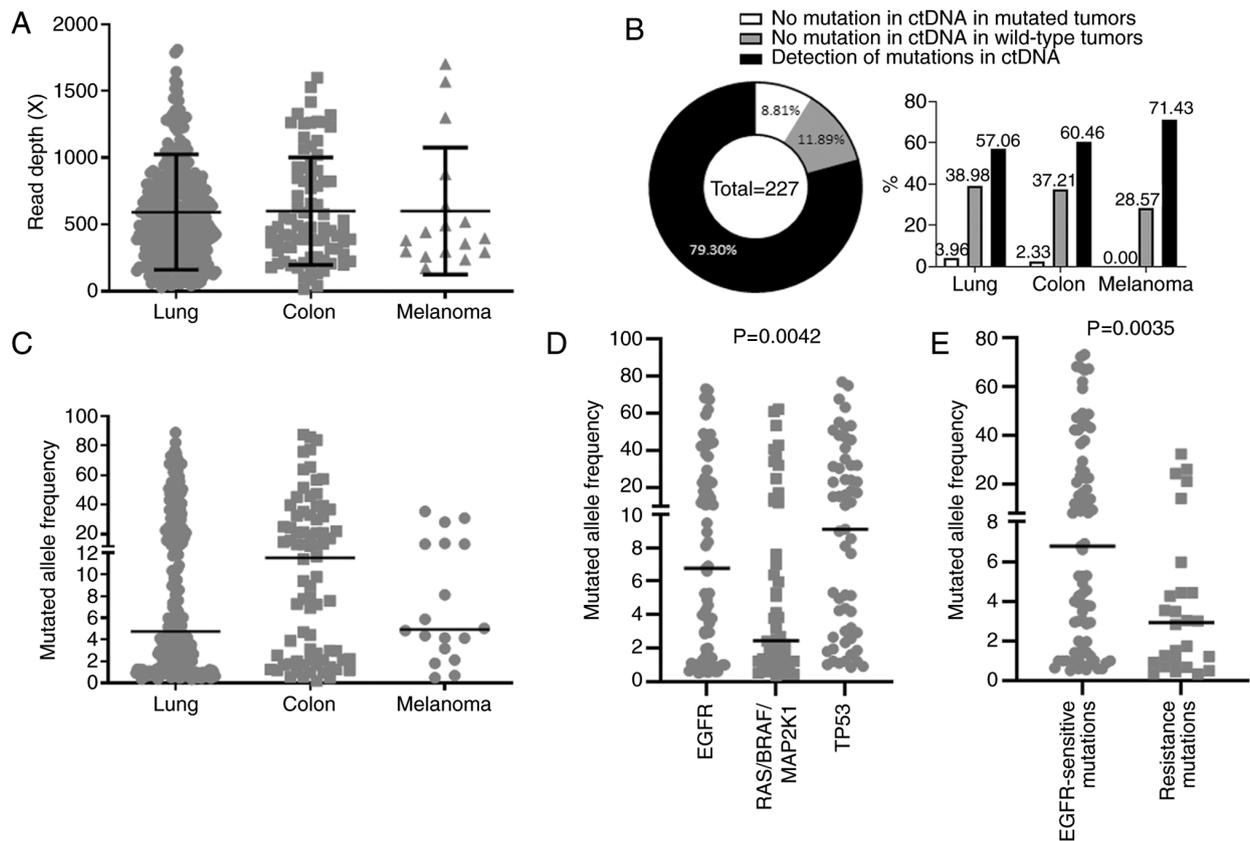


Figure 4. Performance of our custom next-generation sequencing panel in liquid biopsies. (A) Read depth coverage obtained for liquid biopsies of lung and colorectal cancers, and of melanomas. (B) Left, detection of variants in 227 liquid biopsies compared with results obtained for matched tumor samples; right, representation of these 227 liquid biopsies depending on their origin. (C) Mutant allele frequencies obtained for liquid biopsies from patients with lung cancer, colorectal cancer and melanoma. (D) Mutant allele frequencies for *EGFR* variants, for *KRAS*, *NRAS*, *BRAF* and *MAP2K1* (*RAS/BRAF/MAP2K1*) variants, as well as *TP53* variants in liquid biopsies from patients with lung cancer. (E) Mutant allele frequencies in liquid biopsies from *EGFR*-mutant lung cancers for variants sensitizing to *EGFR* tyrosine kinase inhibitor and those inducing resistance to this treatment. ctDNA, circulating tumor DNA.

Discussion

The increase in the incidence of solid cancers combined with the development of targeted therapies require molecular diagnosis laboratories to genotype an increasing number of genes in increasing numbers of samples, with more or less redundant targets. A possibility to satisfy this need in a reduced amount of time emerged with the application of NGS which enables molecular biologists to analyze numerous targets for numerous patients simultaneously and within timing compatible with patient care. However, providing reliable results quickly, at an affordable cost, and in accordance with quality standards remains one of the major challenges of molecular diagnostics. In order to tackle this challenge, certain manufacturers developed commercial kits for analyzing dozens of genes (from 20 to 50, or even more) known to be involved in the development of solid and liquid cancers. These gene panels are usually based on capture technology and focus on hotspot-containing regions of selected genes. These selections do not appear to be compatible with clinical practice (30). In fact, routine molecular diagnosis should first and foremost help guide decisions on prescribing approved treatments. Therefore, the laboratories do not have to screen for other targets which would be necessary for inclusion in clinical trials or for off-label prescription. Both of these activities require specific knowledge and expertise in a regulated environment (clinical

trials), which is not necessary in the context of daily routine diagnostic services. For all these reasons, a ten-gene custom NGS panel was developed for routine diagnosis of four cancer types including lung cancers, colorectal cancers, melanomas and GISTs. In the present study, the design and performance of this panel was described. Our three-year experience of using it routinely in our diagnostic practice was also reported.

Our panel was validated on 15 items according to the ISO15189 standards, obtaining the accreditation required for its use in routine diagnostic laboratories in France. It exhibited very favorable analytical performances, with the 100% accuracy for genotyping hotspot regions with a relatively low coverage. It was revealed that a minimal coverage of 80X on each nucleotide was sufficient to obtain a sensitivity and specificity of 100% in our experimental conditions (tumor cell content >5%). In real life, and particularly for liquid biopsies and hotspot regions, the minimal coverage obtained is widely over 80X. Real life sensitivity obtained for the analysis of liquid biopsies was 91.19%, which remains higher than that reported in recent studies (31-33). It is important to note that hotspot visualization on raw data for liquid biopsies is essential to obtain such sensitivity.

The analysis of the whole coding sequences of ten genes with a therapeutic potential revealed that 73.22% of the observed variants (frequency <1% in the general population) were either benign (5.68%), or of unknown significance (67.54%).

Table III. Fifteen items assessed and the results obtained for our next-generation sequencing method validation in accordance with the ISO15189 standard.

Item tested	Result
Repeatability	Coefficient of variation < or =20.2%
Intermediate fidelity	Coefficient of variation < or =13.5%
Rightness	-18% < Bias <3%
Accuracy	100% for the genotype; 89% for allelic frequency
Sensitivity	100%
Specificity	100%
Benchmarking	100% concordance
Background	<1%
Coverage	100% of regions of interest
Limit of detection	1% of the mutated allele
Intra-run contamination	No contamination detected
Inter-run contamination	No contamination detected
Robustness	Continuously managed with a positive control
Stability of reagents	All reagents and libraries are stable under our laboratory conditions of use

Consequently, a small proportion of the observed variants could be classified as GoF (16.61%) or LoS variants (9.32%). Given that only GoF variants have a therapeutic impact, only a small number of variants are clinically important, and those are localized in specific exons. A total of 156 exons were analyzed and it was identified that only 37 exons harbored clinically pertinent variants. Nevertheless, despite the low impact of non-hotspot regions, it is important to cover them to accommodate for future applications of gene amplification analysis. Indeed, it appears that copy number amplifications could be useful as markers predicting response to treatments (34,35) or as theranostic biomarkers (8,36,37). Therefore, the analysis of the entire gene, or at least of its whole coding sequence, warrants a more reliable analysis.

It was decided to not include intron regions specific to fusion of *ALK*, *ROS1* or *RET* in the panel. In fact, research of fusions from DNA requires the knowledge of the specific partners and their specific breakpoints, rendering it impossible to detect 100% of fusions. It is considered that targeted RNAseq is more appropriate for fusion detection. Nevertheless, it is totally possible to add these intron sequences and/or other coding regions of new genes to the panel. New target genes could be identified at any time depending on the results of clinical trials. Based on this fact, our research group selected capture technology that allows the addition of new probes without disturbing the specificity of the validated panel backbone.

Through the analysis of lung tumor samples, hotspot variants were detected in ~74% of samples, with more than a half of them harboring a variant in the *KRAS* (45%) or *BRAF* gene (4%), markedly higher than described to date (38,39). The analysis of *MAP2K1* and *NRAS* revealed that 2% of the tumors

harbored a GoF mutation that could be targetable by MEK inhibitors (40). Variants in tyrosine kinase domain receptors were found in 19% of patients, with the highest prevalence of *EGFR* mutations (15%), the latter being slightly higher than expected for Caucasian patients with lung cancer based on literature data (41,42).

In colorectal cancer, *KRAS* has been reported as the most often mutated gene (40%), followed by *BRAF* (10%), and *NRAS* (4%) (43). With our panel, it was found that 50% of colorectal cancers were *KRAS*-mutant and 13% had variants in *BRAF*, indicating that our panel may be more sensitive in detecting variants in these genes. The *NRAS* mutation prevalence identified with our panel (3.8%) was very close to that reported in the literature (4%) (43,44). Notably, ~2% of the analyzed colorectal cancers had GoF variants in the *MAP2K1* gene, which may give rise to resistance to anti-EGFR therapies (44). Finally, due to the inclusion of the *ERBB2* gene in the panel it was revealed that 3% of colorectal cancers had a GoF variant, a finding which could help identify new therapeutic strategies for these patients (45).

In melanoma, a prevalence of *BRAF* mutations was obtained in accordance with that reported in a recent study (38.61% of mutant tumors), whereas the prevalence of *NRAS* variants (26.58%) was higher than reported (16.4%), and that of *KIT* variants was lower (3.16% vs. 10%) (46). The low prevalence of *KIT* variants could be explained by the lower number of mucosal melanomas in our series, whereas the higher prevalence of *NRAS* variants may be explained by the fact that our patients were mostly Caucasians. In fact, *NRAS* variants were more often reported in populations from Italy, Sweden, Spain, and the USA (46), predominantly Caucasian, similar to the population analyzed in the present study.

Finally, even if the number of GISTs included in our analysis was quite low, it is noteworthy that 83% of them harbored a *KIT* or a *PDGFRA* GoF variant, and that *PDGFRA* variants occurred in ~30% of *KIT*-wild-type locally-advanced GISTs, as described in previous studies (47,48). In contrast to other analyzed cancers, GISTs did not harbor any GoF variants in other genes of the panel. LoF variants were also found in certain oncogenes. *BRAF* LoF variants have already been described as inducing constitutive activation of CRAF and downstream protein kinases (49). However, in our series, LoF variants were also present in tyrosine kinase receptors. The absence of loss of heterozygosity detection did not enable us to conclude on the complete loss of the protein. To date, no study had investigated the impact of such variants on tumor cells. These may be merely passenger variants but they may also have an activating impact on tumor cells. The latter hypothesis is supported by the fact that certain variants are clearly associated with particular cancer types. For example, *EGFR* mutations generating a stop codon in the tyrosine kinase domain were only detected in lung cancers, whereas *MET* and *PDGFRA* LoF variants were mainly present in colon cancers. Further studies are required to clarify this issue.

Our clinical practice demonstrated that processing liquid biopsies at the same time as solid samples produced favorable analytical performances (owing to the reading of hotspots from raw data and the use of LoF variants of the *TP53* gene). In addition, in liquid biopsies median mutant allele frequencies differed between tumor types. They were significantly

Table IV. Distribution of the four classes of genetic variants in the ten genes included in our next-generation sequencing panel for each tumor type.

Variant significance	Lung cancer (n=1299) (%)	Colon cancer (n=790) (%)	Melanoma (n=158) (%)	Gastrointestinal stromal tumor (n=42) (%)
<i>ALK</i> variants				
Benign	9 (0.69)	10 (1.27)	1 (0.63)	0 (0)
Unknown	96 (7.39)	68 (8.61)	23 (15.46)	1 (2.38)
Gain-of-function	1 (0.08)	0 (0)	0 (0)	0 (0)
Loss-of-function	12 (0.92)	11 (1.39)	1 (0.63)	0 (0)
<i>BRAF</i> variants				
Benign	2 (0.15)	2 (0.25)	2 (1.27)	0 (0)
Unknown	39 (3)	16 (2.03)	13 (8.23)	2 (4.76)
Gain-of-function	54 (4.16)	103 (13.04)	61 (38.61)	0 (0)
Loss-of-function	25 (1.92)	24 (3.04)	9 (5.70)	0 (0)
<i>EGFR</i> variants				
Benign	10 (0.77)	3 (0.38)	1 (0.63)	0 (0)
Unknown	59 (4.54)	36 (4.56)	20 (12.66)	2 (4.76)
Gain-of-function	196 (15.09)	6 (0.76)	0 (0)	0 (0)
Loss-of-function	4 (0.31)	3 (0.38)	1 (0.63)	0 (0)
<i>ERBB2</i> variants				
Benign	54 (4.16)	33 (4.18)	3 (1.9)	2 (4.76)
Unknown	53 (4.08)	26 (3.29)	12 (7.59)	2 (4.76)
Gain-of-function	18 (1.39)	24 (3.04)	0 (0)	0 (0)
Loss-of-function	1 (0.08)	2 (0.25)	0 (0)	0 (0)
<i>KIT</i> variants				
Benign	6 (0.46)	7 (0.89)	0 (0)	0 (0)
Unknown	58 (4.46)	38 (4.81)	9 (5.7)	8 (19.05)
Gain-of-function	2 (0.15)	1 (0.13)	5 (3.16)	31 (73.81)
Loss-of-function	3 (0.23)	6 (0.76)	2 (1.27)	1 (2.38)
<i>KRAS</i> variants				
Benign	2 (0.15)	0 (0)	1 (0.63)	0 (0)
Unknown	5 (0.38)	3 (0.38)	4 (2.53)	0 (0)
Gain-of-function	627 (48.27)	396 (50.13)	9 (5.7)	1 (2.38)
Loss-of-function	0 (0)	2 (0.25)	0 (0)	0 (0)
<i>MAP2K1</i> variants				
Benign	1 (0.08)	0 (0)	0 (0)	0 (0)
Unknown	17 (1.31)	5 (0.63)	1 (0.63)	0 (0)
Gain-of-function	9 (0.69)	8 (1.01)	3 (1.9)	0 (0)
Loss-of-function	2 (0.15)	0 (0)	0 (0)	0 (0)
<i>MET</i> variants				
Benign	43 (3.31)	16 (2.03)	2 (1.27)	1 (2.38)
Unknown	72 (5.54)	42 (5.32)	25 (15.82)	0 (0)
Gain-of-function	38 (2.93)	1 (0.13)	1 (0.63)	0 (0)
Loss-of-function	6 (0.46)	15 (1.90)	3 (1.9)	0 (0)
<i>NRAS</i> variants				
Benign	1 (0.08)	0 (0)	0 (0)	0 (0)
Unknown	11 (0.85)	2 (0.25)	3 (1.9)	0 (0)
Gain-of-function	19 (1.46)	30 (3.80)	42 (26.58)	0 (0)
Loss-of-function	0 (0)	2 (0.25)	1 (0.63)	0 (0)
<i>PDGFRA</i> variants				
Benign	58 (4.46)	46 (5.82)	6 (3.8)	1 (2.38)
Unknown	65 (5)	48 (6.08)	15 (9.49)	0 (0)
Gain-of-function	0 (0)	1 (0.13)	0 (0)	4 (9.52)
Loss-of-function	4 (0.31)	10 (1.27)	0 (0)	0 (0)

higher for colorectal cancers than for lung cancers or melanomas, suggesting that progressing colorectal cancer may release more cell-free DNA. When focusing on lung cancer samples, it was also found that mutated allele frequencies were different between altered genes. *TP53* and *EGFR* variants had significantly higher mutated allele frequencies than variants in the *RAS/BRAF/MAP2K1* genes. Finally, it was also found that in lung cancers progressing under TKI, variants inducing resistance to treatment had significantly lower allele frequencies than the original sensitizing variants.

In conclusion, our three-year practical experience using a custom ten-gene NGS panel for the molecular diagnosis of four solid cancers produced very positive results. It was demonstrated that this panel has favorable analytical performances and can be used for analyzing not only solid but also liquid biopsies. With our work process, it was possible to release clinical reports in less than five working days from the receipt of samples. The use of our panel can provide for a more relevant biomarker analysis and could be an effective way to counter the problem of low molecular diagnosis. The latter should not be underestimated. It was reported that less than 50% of patients had no molecular results before receiving the first line of treatment (50). In our opinion, the use of relatively small dedicated NGS panels is the optimal choice to rapidly obtain relevant results before first-line treatment. Such a method should also be developed for routine diagnosis of other cancer types, whereas the use of large panels should be reserved for patients in therapeutic failure.

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Availability of data and materials

Genomic data could be shared upon reasonable request to the corresponding author in accordance to French law for genomic data.

Authors' contributions

SC designed the panel and performed the experiments. AB, MC, ACh, AG and MJ performed the experiments. HM and CT performed bioinformatics analyses. FB, CCB and LA qualified solid samples. JA, ACo, VD and VG interpreted the routine results. RB designed the panel, interpreted the routine results, analyzed the data, designed the study and wrote the manuscript. RB and VG confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study on patient samples was conducted in accordance with the Declaration of Helsinki and was approved

(approval no. 00010311) by the Ethics Committee of the Georges-François Leclerc Cancer Center (Dijon, France) and by the Consultative Committee of Burgundy (Dijon, France) for the Protection of Persons Participating in Biomedical Research (Comité Consultatif de Protection des Personnes en Recherche Biomédicale de Bourgogne). Written informed consent was provided by all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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