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Impact and Reproducibility of In-House Targeted Next-Generation Sequencing Biomarker Testing in Non—Small-Cell Lung Cancer

An Italian Multi-Institutional Experience

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Next-generation sequencing (NGS) allows the detection of multiple genetic targets in different tumor types. This study aimed to confirm the benefits of implementing in-house NGS testing for non—small-cell lung cancer (NSCLC) samples in molecular pathology laboratories. A multi-institutional study was conducted to evaluate the analytical performance, turnaround time, and feasibility of in-house NGS testing of 50 genes from 283 NSCLC samples. The first phase was a retrospective study with interlaboratory testing (21 samples), and the second phase was a prospective study with intralaboratory testing (262 samples). The retrospective study showed a 100% sequencing success rate for DNA and RNA, high interlaboratory concordance (95.2%), and a strong correlation ($R^2 = 0.94$) between observed and expected single-nucleotide variant/insertion and/or deletion variant allele fraction. The prospective study showed a sequencing success rate of 99.2% for DNA and 98% for RNA. NGS identified 285 relevant variants (81.1% single-nucleotide variants/insertion and/or deletion variants, 9.8% copy number variants, and 9.1% gene fusions). Co-mutations with potential clinical relevance were detected in 20.5% of samples positive for the main oncogenic drivers in NSCLC. Additionally, 11% of samples wild type for the main oncogenic drivers carried alterations in other relevant genes. The in-house NGS testing had a median turnaround time from sample processing to molecular report of 4 days. This study demonstrates the advantages of implementing in-house NGS testing in molecular pathology laboratories. *(J Mol Diagn 2025, ■: 1—12; https://doi.org/10.1016/j.jmoldx.2025.02.001)*

The number of targeted therapies for non—small-cell lung cancer (NSCLC) is ever expanding, demanding an increasing number of predictive biomarker tests.[1] As a result, the number of mandatory molecular targets that must be analyzed to comprehensively characterize samples from patients with advanced NSCLC has increased significantly.[2] This advancement has improved the clinical management of patients with advanced NSCLC[2] and increased overall survival rates.[3,4] The National Comprehensive Cancer Network (NCCN),[5,6] the European Society for Medical Oncology (ESMO) guidelines, and, in particular, the ESMO Scale for Clinical Actionability of Molecular Targets (ESCAT) classification[7,8] provide a systematic framework for ranking molecular targets based on clinical evidence of their actionability. The list of ESMO Scale for Clinical Actionability of Molecular Targets tier I to II actionable genetic variants for NSCLC currently includes single-nucleotide variants (SNVs) and insertion and/or deletion variants (indels) of *EGFR*, *BRAF*, *KRAS*, and *ERBB2*, fusions of

Reagents for the interlaboratory validation phase 1 of the study were provided by agreement with Thermo Fisher Scientific.

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ROS1, *ALK*, *NTRK1/2/3*, and *RET*, and *MET* exon 14 skipping mutations. Other biomarkers, such as *MET* and *ERBB2* amplifications, have therapeutic potential and are expected to be soon considered targetable genes according to the ESMO Scale for Clinical Actionability of Molecular Targets guidelines.[9]

Ongoing identification of multiple targets is also important in early-stage NSCLC, where new clinical recommendations for adjuvant treatment have recently been introduced.[10]

In the field of precision medicine, next-generation sequencing (NGS) tests are the most important tool for implementing personalized therapy. These tests are highly sensitive and can provide the genetic characterization of multiple biomarkers simultaneously and in a short time. Nevertheless, not all diagnostic laboratories have implemented NGS platforms. This is mainly because of the high costs of setting up the laboratory and performing the tests, as well as the high demands on the molecular biology expertise of the staff.[11] Current genomic testing for NSCLC in Italy uses multigene DNA- and RNA-based small panels (<50 genes). However, these RNA-based panels, which are commonly used in diagnostic laboratories to identify gene fusions, require high-quality RNA that must be verified before NGS testing. This verification often reveals that the quality and integrity of the RNA does not meet the requirements of the assay, resulting in a high RNA failure rate. Therefore, multiple-gene RNA analysis is complemented by single-gene testing methods, such as real-time PCR, fluorescence *in situ* hybridization (FISH), and immunohistochemistry (IHC).[12] Sequential identification of single-gene mutations is time-consuming and can potentially exhaust the available tissue before all relevant biomarkers are measured. On the other hand, NGS performed using gene panels suitable for routine diagnostics offers a cost-effective solution with high sensitivity and competitive response turnaround time (TAT) for result delivery, allowing accurate identification of all biomarkers, on both DNA and RNA, through a single analysis workflow.[13−17]

NGS offers solutions to the existing challenges of low tumor cell content and small sample size.[13,18−21] Therefore, it provides a sensitive and rapid method for identifying specific mutations that could enable the use of clinically optimized targeted therapy regimens for individual patients. Indeed, an association between comprehensive molecular genotyping and improved overall survival has been demonstrated in patients with NSCLC.[22]

Optimizing institutional workflows and implementing NGS facility networks could further reduce delays in routine diagnostics. Ideally, pathologists should have access to comprehensive genomic results within a few days. Bringing NGS analysis in-house, simplifying current procedures, and facilitating adoption by local hospitals would be a fundamental change in the field, enabling faster TAT, better control of the pre-analytical phase of sample preparation, and improved patient care by selecting the most appropriate targeted therapies. This approach is feasible and cost-effective for the detection of DNA and RNA variants in formalin-fixed, paraffin-embedded (FFPE) samples and shows 100% concordance with the most commonly used comparative methods (real-time PCR or FISH).[23,24] Therefore, it could enable a comprehensive assessment of clinically relevant tumor-associated genetic alterations at the tissue level.[25] Moreover, NGS testing has been shown to be feasible for the detection of DNA variants in liquid biopsy samples,[26] thus providing an alternative or complementary approach for the assessment of molecular biomarkers in tumor progression or in cases where tissue material is unsuitable for molecular analyses. In addition to genes that are targets for first-line treatment of NSCLC, expanded target NGS panels are also investigating genes that, if co-mutated, may affect response to targeted therapy or represent additional targets for second-line therapies. For example, *TP53* mutations have been shown to negatively affect response to tyrosine kinase inhibitors (TKIs) in *EGFR*-mutated patients,[27] suggesting the need for new combination therapies.[28]

Among the NGS platforms and commercially available panels for clinical research, the Ion Torrent Genexus System (Thermo Fisher Scientific, Waltham, MA) in combination with the Oncomine Precision Assay GX (OPA; Thermo Fisher Scientific; both products for research use only, not for diagnostic use) enables the automation of targeted NGS workflows using various biological materials, from FFPE tissue biopsies to cell-free total nucleic acids from liquid biopsies, allowing results to be obtained even from small and low-quality samples.[13]

This study describes the experience of three Italian centers with in-house targeted NGS using a fully automated and end-to-end NGS solution, including an assay that interrogates all ESMO Scale for Clinical Actionability of Molecular Targets I/II genetic variants of NSCLC. The evaluation included a retrospective and a prospective step with the determination of analytical performance, TAT, and feasibility of the assay using NSCLC samples.

Materials and Methods

Research Study Sites and Their Laboratory Experience Before and After the Introduction of the Ion Torrent Genexus System

The study was conducted in three centers (A, B, and C) in Italy: center A, Fondazione IRCCS San Gerardo dei Tintori, Monza; center B, IRCCS Regina Elena National Cancer Institute, Rome; and center C, Pathology Unit at the University Hospital of San Luigi Gonzaga, Orbassano, Turin.

The research protocol was reviewed and approved by all Ethics Committees of the centers (center A cod. 3585 RAS ATLAS, 24.10.2022; center B cod. R.S. N.1364/20; center C cod. N.73/2018). The experience of each center involved in the study before and after the implementation of NGS testing

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Table 1 Experience of the Three Centers Before and After Using the Oncomine Precision Assay (OPA) on the Ion Torrent Genexus System

Center A	Before Genexus (OFA on Ion PGM System)	From OPA on Genexus (1 month of experience before the study to May 2022)
Number of NSCLC samples analyzed per month (average in 1 year)	16 Cases	20 Cases
Number of personnel dedicated to tumor molecular profiling	2	2
TAT* per sample	12 Working days	6 Working days
Center B	**Before Genexus (OFA on Ion S5 System)**	**From OPA on Genexus (8 months experience before the study to October 2021)**
Number of NSCLC samples analyzed per month (average in 1 year)	50 Cases	70 Cases
Number of personnel dedicated to tumor molecular profiling	5	3
TAT* per sample	10 Working days	3 Working days
Center C	**Before Genexus (OFA on Ion S5 System)**	**From OPA + Genexus (7 months experience before the study to November 2021)**
Number of NSCLC samples analyzed per month (average in 1 year)	55 Cases	73 Cases
Number of personnel dedicated to tumor molecular profiling	3	3
TAT* per sample	10 Working days	4 Working days

Centers: center A, Fondazione IRCCS San Gerardo dei Tintori (Monza, Italy); center B, IRCCS, Regina Elena National Cancer Institute (Rome, Italy); and center C, Pathology Unit at the University Hospital of San Luigi Gonzaga (Orbassano, Turin, Italy). Ion PGM System and Ion S5 System are products of Thermo Fisher Scientific.

NSCLC, non—small-cell lung cancer; OFA, Oncomine Focus Assay; PGM, Personal Genome Machine; TAT, turnaround time.

*TAT: the time from the start of sample processing to the generation of the next-generation sequencing molecular report. To calculate TAT using OPA on the Ion Torrent Genexus System, the median TAT of samples tested during phase 2 of the study was determined for each center.

with the Genexus System and OPA panel in terms of the number of samples analyzed per month, the number of personnel dedicated to molecular diagnosis, and TAT for NGS testing report is summarized in Table 1. TAT was defined as the time between the start of sample processing and the generation of the NGS molecular report. This time frame included the steps of microdissection or macrodissection, deparaffinization, nucleic acid extraction, sequencing, and the generation of the NGS molecular report.

Tumor Samples Used in Phase 1 and 2 of the Study

This study comprised two phases. Phase 1 was a retrospective analysis of 21 NSCLC FFPE samples from each center to perform an interlaboratory NGS comparison. Eight samples were selected by center A, six samples by center B, and seven samples by center C. The 21 cases collected were selected to cover, in at least one case, all molecular targets that currently define first-line treatment according to the latest ESMO guidelines[8] (*EGFR*, *KRAS* G12C, *BRAF* V600E, *ALK*, *ROS1*, *RET*, *MET*, and *NTRKs*, except *ERBB2*). One case was wild type for the genes covered by the panel and was used as a negative control. The NSCLC samples selected by each center were exchanged among centers and tested by all. Each participating center prepared two sets of unstained serial sections for each case and sent them to the other two centers so that each center could test each sample under its own preanalytical conditions of tumor cell enrichment and nucleic acid extraction. Samples were precharacterized using the Oncomine Focus Assay (Thermo Fisher Scientific; for research use only, not for diagnostic use), real-time PCR, IHC, and/or FISH, according to each center's testing protocols.

Phase 2 of the study was a prospective analysis of 262 NSCLC samples collected between June 1 and September 15, 2022. All cases were sequential and unselected, with >90% of cases tested on the basis of a molecular profiling request and a few cases tested reflexively because they were classified as advanced NSCLC. Sample characteristics are summarized in Table 2. A total of 53 samples were collected

Table 2 Sample Characteristics

Center	Sample sources, %	Sample type, %
Center A	In-house: 95 External: 5	Cytologic/biopsy: 87 Surgical: 13
Center B	In-house: 46 External: 54	Cytologic/biopsy: 78 Surgical: 22
Center C	In-house: 50 External: 50	Cytologic/biopsy: 92 Surgical: 8

Table 3 Pathologic Features of the Study Samples

Study	Characteristics		n (%)
Retrospective (n = 21)	Histology	Adenocarcinoma	19 (90.4)
		NSCLC-NOS	1 (4.8)
		Others	1 (4.8)
	pTNM stage	Stage I	6 (28.6)
		Stage II	2 (9.5)
		Stage III	3 (14.3)
		Stage IV	10 (47.6)
Prospective (n = 262)	Histology	Adenocarcinoma	228 (87.0)
		Squamous cell carcinoma	13 (5.0)
		NSCLC-NOS	13 (5.0)
		Others	8 (3.0)
	pTNM stage	Stage I	19 (7.3)
		Stage II	5 (1.9)
		Stage III	24 (9.2)
		Stage IV	167 (63.7)
		NA	47 (17.9)

NA, not applicable; NSCLC-NOS, non—small-cell lung cancer not otherwise specified; pTNM, pathologic tumor-node-metastasis.

by center A, 103 samples by center B, and 106 samples by center C. The tissue samples were 95% from in-house and 5% from external sources for center A; 46% from in-house and 54% from external sources for center B; and 50% from in-house and 50% from external sources for center C. In 92% of cases in center C, 87% in center A, and 78% in center B, the type of sample was cytology or biopsy specimens. The remaining cases were surgical specimens in all centers. In all centers, tumor cell enrichment was performed as either macrodissection from unstained slides (centers A and B) or manual microdissection from stained slides with microscopy support (center C).[29] After tumor cell enrichment, the percentage of tumor cells in all centers ranged from 5% to 90%, with most samples containing >30% tumor cells. The proportion of cases with a tumor cell content <30% was 8.4% (22/262), of which 6, 13, and 3 cases were in centers A, B, and C, respectively. The histopathologic data and tumor stages of the phase 1 and phase 2 cases are described in Table 3. The high proportion of cases that were cytologically evaluated on the basis of presurgical material justifies the classification of 14 cases as NSCLC, not otherwise specified (ie, without morphologic or immunophenotypical signs of squamous, glandular, or neuroendocrine differentiation).

Nucleic Acid Extraction

Each of the centers performed a similar workflow with the described modifications. Each participating center prepared two sets of unstained serial sections of FFPE tumor samples for each case to distribute to the other two participating centers. Depending on the cellularity of the sample tested, slides or curls (5 to 8 μm thick) were used to detect DNA and RNA variants in FFPE material (ie, four slides for DNA and four or five slides for RNA for resected tumor samples). For small biopsies with a tumor cell content of <20%, the number of slides was increased to 8−10, depending on the availability of tissue in the block. Tumor cell enrichment was performed through dissection, as described in the previous section, for tumor samples in phase 2 of the study. In center A, nucleic acid purification and quantification were performed simultaneously and automatically starting from the same FFPE slides using the Ion Torrent Genexus Purification System (Thermo Fisher Scientific; for research use only, not for diagnostic use), equipped with a fluorometer that analyzes the extracted nucleic acid after the isolation step. In the other centers, the Genexus Purification System was not available. DNA and RNA were isolated sequentially from tissue sections using, in center B, the QIAamp DNA Kit (Qiagen, Milan, Italy) and the MagCore Total RNA FFPE Kit with the MagCore Automated Nucleic Acid Extractor (RBC Bioscience, Jesi, Italy). In center C, the Maxwell CSC DNA FFPE Kit and the Maxwell CSC RNA FFPE Kit (Promega Corp., Madison, WI) were used. Concentrations of DNA and RNA were determined by fluorimetric quantification using the Qubit DNA dsDNA BR Assay Kit and the Qubit RNA BR Assay Kit in center B (Thermo Fisher Scientific), and the Quantifluor System (Promega Corp.) at center C.

Genomic Profiling by NGS

The Oncomine Precision Assay was used to analyze 50 genes (Supplemental Table S1) covering 2769 unique variants, including hotspot mutations, copy number variants (CNVs), gene fusions (including those with unknown partners by assessing exon tiling imbalance), and splice site variants. A total of approximately 10 ng of genomic material was used to generate libraries using the Ion Torrent Genexus System. The Ion Torrent Genexus System was used for templating and sequencing of all samples. The plexing of the samples was performed according to the manufacturer's instructions. The samples were sequenced using GX5 chips (Thermo Fisher Scientific; for research use only and not for diagnostic use). NGS data were analyzed using Genexus software version 6.2 (Thermo Fisher Scientific). The Torrent Browser of Torrent Suite software version 6.2.1 (Thermo Fisher Scientific) was used for initial quality control, including chip loading density, median read length, and number of mapped reads. The manufacturer's limit of detection for variants in FFPE samples, set at >5%, was applied consistently across all centers. Variants were reported following the NGS guidelines of the Italian Society of Pathological Anatomy and Cytology [*https://www.siapec.it* (Italian), last accessed January 6 to September 15, 2022], and were classified as pathogenic, likely pathogenic, or variants of uncertain significance. Potentially clinically relevant mutations identified with an allele fraction <5% were validated at each center using orthogonal methods.

Orthogonal Methods for the Validation of NGS Results

NGS results were validated according to the clinical workflow established in each center. In center A, gene variants were confirmed by real-time PCR (Easy EGFR, KRAS, BRAF kits; Diatech Pharmacogenetics, Jesi, Italy) or Sanger sequencing if the allele fraction was >15%. Evaluation by IHC was performed in selected cases for ALK tyrosine kinase receptor (ALK; clone D5F3) and serine/threonine-protein kinase B-raf (BRAF; clone VE1) on the Dako Omnis platform (Agilent Technologies, Santa Clara, CA).

In center B, all cases underwent routine IHC evaluation of expression imbalance for ALK (clone D5F3; Ventana/Roche Diagnostics, Indianapolis, IN) and proto-oncogene tyrosine-protein kinase ROS (ROS1; clone D4D6; Cell Signaling Technology, Danvers, MA). In addition, the Idylla GeneFusion Assay (Biocartis NV, Mechelen, Belgium) was used as a routine orthogonal test in scenarios where read counts for a targeted fusion are just above the threshold to avoid the risk of false positives, such as those resulting from contamination. The Idylla GeneFusion Assay has also been used in cases where results were inconsistent with the IHC test or when RNA sequencing was considered suboptimal, thus, to reduce the presence of false negatives. In selected cases, gene variants were validated by Idylla EGFR, KRAS, BRAF mutation tests (Biocartis NV). *MET*, *EGFR*, and *ERBB2* copy number gain detected by OPA was confirmed by FISH as part of the routine procedure using the ZytoLight Dual Color Probes: SPEC MET/CEN 7, SPEC EGFR/CEN 7, and SPEC ERBB2/CEN 17 (Zytovision GmbH, Bremerhaven, Germany). In center C, gene variants in selected cases (ie, low allele fraction, such as the discrepant case in phase 1) were validated by a real-time PCR approach using the EasyPGX ready kit for *KRAS*, *BRAF*, *EGFR* (all from Diatech Pharmacogenetics). Gene fusions were validated either with the EasyPGX ready kit for *ALK*, *ROS1*, *RET*, and *MET*, or with a FISH or IHC approach. For *ALK* testing, IHC was performed using the 5A4 clone (Novocastra, Leica Biosystems, Nussloch, Germany), whereas FISH was performed using the Vysis ALK Break Apart Kit (Vysis, Abbott Molecular Inc., Des Plaines, IL). *ROS1* was tested by IHC with the D4D6 clone (Cell Signaling Technology) and by FISH with the ZytoLight SPEC ROS1 Dual Color Break Apart Probe (Zytovision GmbH). *NTRK* gene fusions were validated by IHC using the EPR17341 clone (Abcam PLC, Cambridge, UK) and by FISH using the NTRK1 Break Apart Probe (locus 1q23.1; Empire Genomics, Buffalo, NY). In each FISH test, the sample was classified as positive if an altered signal was present in >15% of the tumor cells.

Statistical Analysis

Data analysis was performed using Microsoft Office Excel software version 2305 (Microsoft Corp., Redmond, WA) and GraphPad Prism 9.5.1 (GraphPad Software Inc., Boston, MA). Pearson correlation coefficient was calculated to measure the linear relationship between the variables. Data visualization was performed using Adobe Illustrator version 27.4 (Adobe Systems, San Jose, CA).

Results

Phase 1 Study

The phase 1 study was designed to evaluate the accuracy, reproducibility, and interlaboratory biomarker concordance of in-house NGS testing using the OPA and Genexus System. All 21 NSCLC samples were successfully sequenced for DNA and RNA by the three centers (100% sequencing success rate), with basic sequencing metrics exceeding the quality threshold and confirming a high degree of performance uniformity between laboratories (Supplemental Table S2). Detection of SNVs, indels, CNVs, fusions, and interlaboratory concordance are reported in Figure 1. The concordance rate of NGS test results between sender and recipient centers was 95.2% (20/21), with only one sample (A5) showing a partially discordant result (Figure 1, A and B). This sample was classified as wild type by NGS testing in center A (sender) and center B (recipient), but not in center C (recipient). Indeed, center C detected the *EGFR* mutation p.G719A (c.2156G>C) in sample A5 with a low allele fraction (3.5%) and a coverage of 4089 reads (Supplemental Figure S1A). To validate the *EGFR* mutation, real-time PCR analysis was performed in center C, which confirmed the presence of the *EGFR* p.G719A mutation (Supplemental Figure S1B). The sample was obtained from a cell block prepared from a pleural effusion that was positive for adenocarcinoma cells of the lung. The cellularity of the sample was enriched with inflammatory cells (mainly lymphocytes), whereas the neoplastic cells consisted of rare aggregates with a papillary architecture (Figure 1B). Subsequently, the raw data in centers A and B were re-evaluated in Integrative Genomics Viewer (*https://igv.org*) to score the calls in nucleotide position c.2156. For center A, of a total of 2820 reads, allele C was called in 20 reads (1%), allele G in 2793 reads (99%), allele A in 6 reads, and allele T in 1 read. For center B, of a total of 2187 reads, allele C was called in 7 reads (0.32%) and allele G in 2180 reads (99.68%). It is noteworthy that manual microdissection using microscopy assistance was performed only in center C. This approach probably led to a higher enrichment of neoplastic cells and thus enabled the detection of genetic variants with higher frequency.

High interlaboratory reproducibility of allele fractions was observed, with a strong correlation ($R^2 = 0.94$, $P < 0.0001$) between the observed allele fraction of SNV/indel in the recipient centers (mean) and the expected allele fraction determined by the sender center (Figure 1C).

Phase 2 Study

Phase 2 study investigated the applicability of in-house NGS testing using the OPA panel on the Genexus System as a tool for mutation assessment in 262 NSCLC samples and

A

Sender center A	Recipient centers B	Recipient centers C
A1 *EGFR* p.L747_P753delinsSer	Concordant	Concordant
A2 *EGFR* ampl; *TP53* p.V172D	Concordant	Concordant
A3 *KRAS* p.G12D;*TP53* p.R267W	Concordant	Concordant
A4 *EGFR* p.L858R	Concordant	Concordant
A5 WT	Concordant	Partially discordant
A6 *MET*(13)::*MET*(15) fusion	Concordant	Concordant
A7 *KRAS* p.G12C	Concordant	Concordant
A8 *TP53* p.R280I; *PDGFRA* p.R560S	Concordant	Concordant

Sender center B	Recipient centers A	Recipient centers C
B1 *CCD6*(1)::*RET*(12) fusion	Concordant	Concordant
B2 *KRAS* G12C	Concordant	Concordant
B3 *EGFR* Ala767_Val769dup; *TP53* p.R273C	Concordant	Concordant
B4 *CDKN2A* p.G55C; *TP53* p.R283P	Concordant	Concordant
B5 *MET*(13)::*MET*(15) fusion	Concordant	Concordant
B6 *EML4*(13)::*ALK*(20) fusion	Concordant	Concordant

Sender center C	Recipient centers A	Recipient centers B
C1 *BRAF* p.V600E;*MTOR* p.L1460P	Concordant	Concordant
C2 *TP53*(10)::*NTRK1*(12) fusion; *EGFR* ampl;*MET* ampl	Concordant	Concordant
C3 *EML4*(13)::*ALK*(20) fusion	Concordant	Concordant
C4 *CD74*(6)::*ROS1*(34) fusion	Concordant	Concordant
C5 *IDH1* p.R132C	Concordant	Concordant
C6 *FGFR1* ampl;*TP53 p*.P278L; *TP53* p.R196P	Concordant	Concordant
C7 *KIF5B*(15)::*RET*(12) fusion; *TP53* p.E221RfsTer23	Concordant	Concordant

Concordant ■ Partially discordant ■

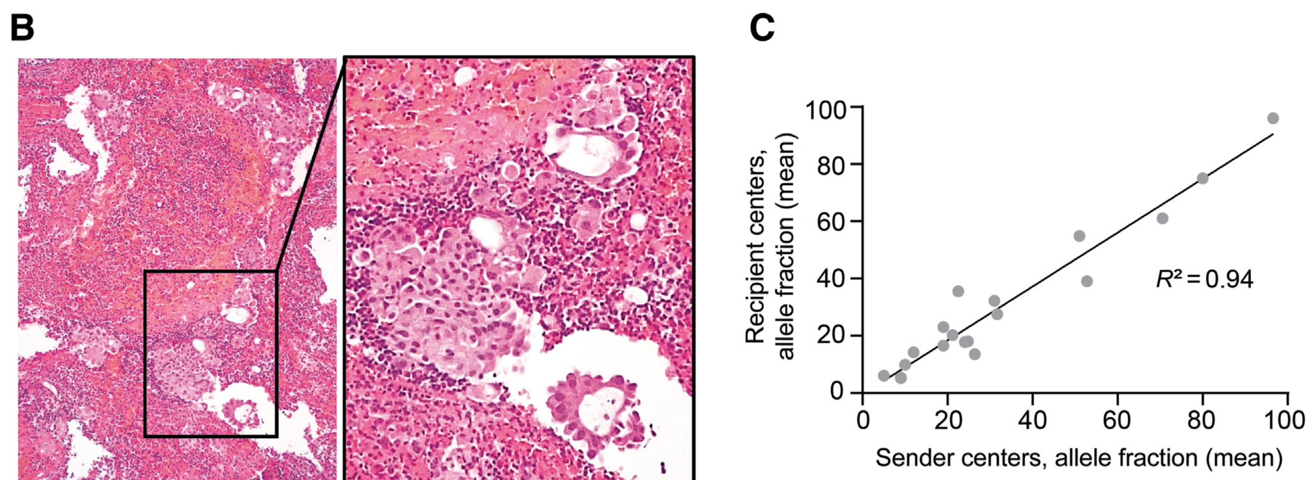

Figure 1 Retrospective study. **A:** Next-generation sequencing results showing the mutations detected in samples analyzed across three independent centers. Complete concordance in mutation detection is indicated in green, whereas partial discordance is shown in orange. **B:** Sample A5, prepared from a cell block of a pleural effusion, contained a predominant inflammatory cell component with small clusters of adenocarcinoma cells. **C:** Correlation analysis of mean allele fraction (single-nucleotide variant/insertion and/or deletion variant) between the sender and recipient centers. Original magnification: ×20 (**B**, **main image**); ×40 (**B**, **enlarged boxed area**). WT, wild type.

evaluated a time-efficient laboratory workflow. The total time from sample processing to generation of the NGS molecular report (TAT), including pre-analytical and post-analytical time, decreased from 10−12 working days when the NGS testing was performed using a non−fully automated NGS platform to 3−6 working days using OPA with the Genexus System in the three centers (Figure 2 and Table 1). Specifically, the median TAT when using OPA to analyze the NSCLC samples in the phase 2 study was 6 working days for center A, 3 working days for center B, and 4 working days for center C (Figure 2). Across all centers, the median TAT was 4 working days.

Of 262 samples tested at the study centers, 99.2% (260/262) had successful DNA NGS results, whereas 98% (257/262) had successful RNA NGS results. NGS failed on external biopsy samples with poor quality and/or low amounts of DNA (<1.2 ng) or RNA (<1.4 ng). In center B, the center with the highest number of external samples, the rate of RNA failure in external samples decreased from 3.5% to 1.5% after the implementation of OPA with the Genexus System.

A total of 285 gene variants were detected in the analyzed NSCLC samples (Figure 3 and Supplemental Table S3). Of the 260 NSCLC samples with valid NGS analysis, 75.0% (195/260) harbored at least one genetic alteration. Among all mutation types, SNV/indel (81.1%, 231/285) was the most frequently detected, followed by CNV (9.8%, 28/285) and gene fusion (9.1%, 26/285). Genetic driver variants, including SNVs, indels, fusions, and CNVs (Figure 3, A−D), were detected as follows: *KRAS* (27.7%, n = 79), *TP53* (21.1%, n = 60), *EGFR* (20.0%, n = 57), *BRAF* (4.9%, n = 14), *PIK3CA* (4.9%, n = 14), *ALK*

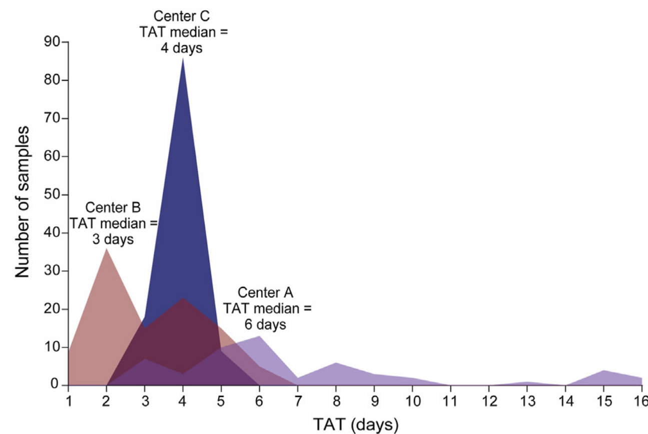

Figure 2 Turnaround time (TAT) for the Oncomine Precision Assay on the Ion Torrent Genexus Integrated Sequencer. The median TAT (number of working days from sample processing to the generation of the molecular next-generation sequencing report) is shown for center A, center B, and center C. Data reflect TAT for the 262 non—small-cell lung cancer samples analyzed in the phase 2 study.

(4.2%, $n = 12$), *MET* (3.4%, $n = 11$), *CDKN2A* (3.2%, $n = 9$), *ERBB2* (2.1%, $n = 6$), *ROS1* (1.8%, $n = 5$), *CTNBB1* (1.4%, $n = 4$), and *RET* (1.1%, $n = 3$), with other alterations detected at frequencies <1% (*CDK4*, *ERBB3*, *FGFR1*, *FGFR3*, *HRAS, IDH2*, *MAP2K1*, *NF1*, *NTRK1*, and *PTEN*). CNVs were detected in several genes with copy number gain, such as *EGFR*, *ERBB2/3*, *FGFR1/3*, *KRAS*, *MET*, and *PIK3CA*. Genes showing copy number loss, such as *CDKN2A*, *NF1*, and *PTEN*, are not shown in Figure 3D. Gene fusions were detected in *ALK* (4.2%, $n = 12$), *MET* (1.8%, $n = 5$), *ROS1* (1.4%, $n = 4$), *RET* (1.1%, $n = 3$), *BRAF*, and *NTRK1*, both <1% ($n = 1$).

The allele fractions in this study ranged from 3.5% to 100%. In cases where center C detected allele fractions <5%, orthogonal methods, such as real-time PCR, PCR, FISH, or IHC, were used to confirm the results. An example of additional evaluation using FISH (Figure 3E) confirmed the copy number gain of *MET* observed with the OPA panel.

As a potential clinical added value of the targeted NGS approach, this study identified 41.1% (107/260) of samples positive for the main genetic drivers in NSCLC (*EGFR*, *KRAS* G12C, *BRAF* V600E, *ALK*, *ROS1*, *RET*, *MET*, *NTRK1/2/3*, and *ERBB2*). Among these, 20.5% (22/107) of samples had co-mutations with potential clinical relevance (Figure 4 and Supplemental Table S4). Supplemental Table S5 lists the genomic alterations that are considered potentially clinically relevant based on the current literature.[30—42] These include additional *EGFR* co-mutations in *EGFR*-mutated cases as well as *ERBB2*, *PI3KCA*, *TP53*, and *KRAS* SNVs/indels. In addition, of the 58.8% (153/260) of NSCLC samples that were negative for mutations in the main driver genes, 11.1% (17/153) were positive with potentially clinically relevant alterations in other genes, including *ERBB2* amplification, *PI3KCA* SNVs, *CDKN2A* loss, *FGFR1* amplification and SNVs, and *MET* amplification (Supplemental Table S5 and Figure 4).

Discussion

The implementation of user-friendly, rapid, automated NGS testing for the detection of genetic alterations and biomarkers in many tumor types is of great value for cancer management. In particular, the ability to provide a short TAT from sample collection to provision of genetic results will enable fast, personalized, and targeted therapeutic interventions, especially in advanced-stage cancers or those with rapid progression.[11] Indeed, the rise of targeted therapies and the identification of new targetable driver mutations in NSCLC have significantly improved patient outcomes,[3] as rapid and reliable detection of multiple mutations in patient samples is critical for the timely identification of personalized and effective treatments.

Evidence-based studies demonstrating quick TAT, reproducibility, adaptability, cost-effectiveness, and consistent performance across different sample types for accurate biomarker identification[43,44] are needed to increase the adoption of NGS in European clinical practice.[45]

Here, several of these aspects were addressed by examining the ability of the OPA panel and the Ion Torrent Genexus System to detect mutations in NSCLC samples. The adopted panel was designed to allow the analysis of all genes currently established as biomarkers for the management of NSCLC. Because of its targeted approach, this strategy did not fulfill the requirements for complete testing. This was especially true for the lack of detection of alterations in tumor suppressor genes (such as in *STK11*, *NF1*, *KEAP1*, or *RB1*), mutational burden, or microsatellite instability. Herein, the main goal was to provide rapid results for timely cancer management. This study demonstrated high reproducibility among centers, consistent performance across all sample types, even with different methods of nucleic acid extraction, and a short TAT of 4 days. This is a significant advantage compared with the TAT reported for sequential analysis of single biomarkers or

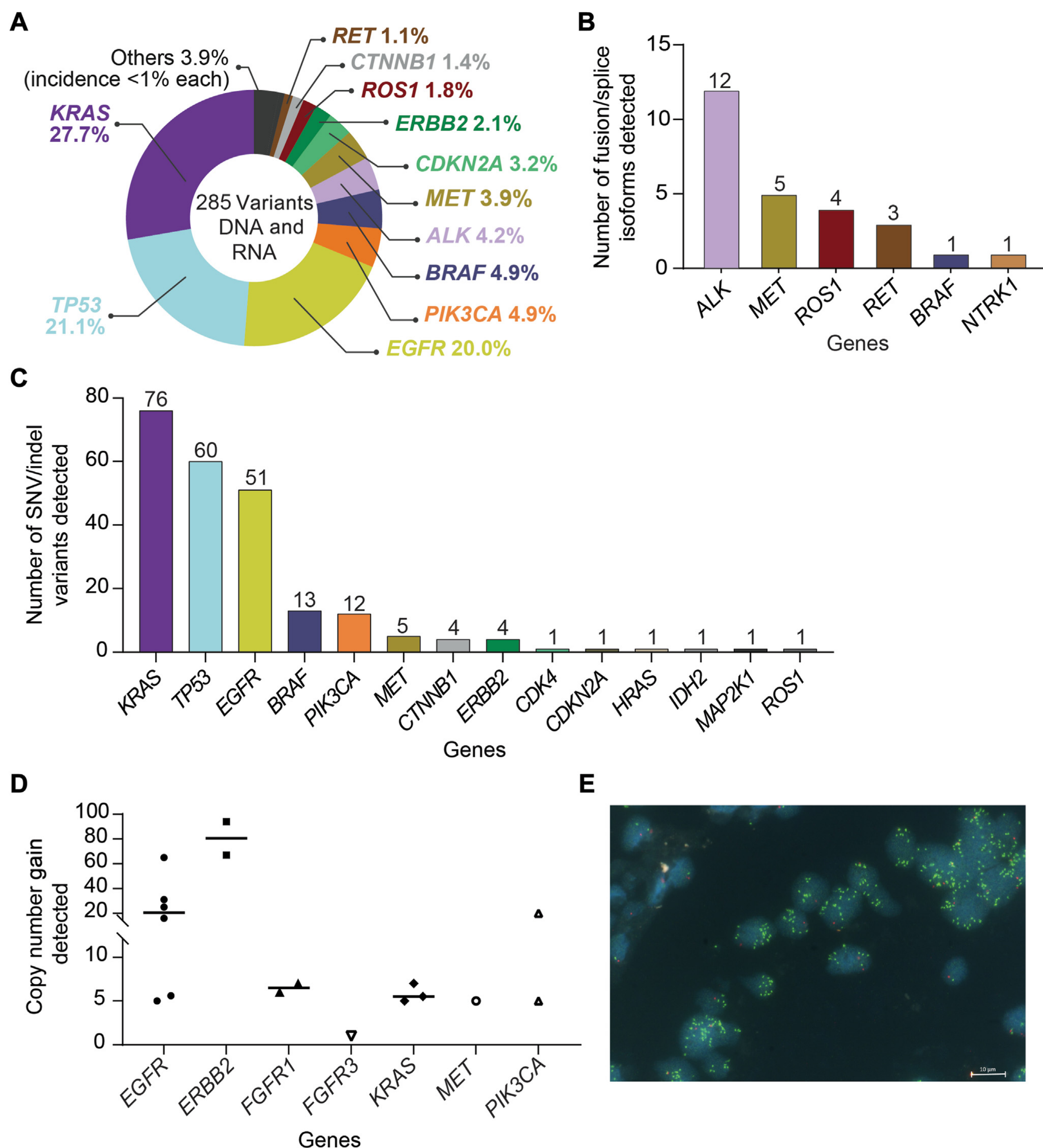

Figure 3 Prospective study: genomic profiling of non−small-cell lung cancer. Variants are shown to illustrate the range of genetic changes detectable using the Oncomine Precision Assay (OPA) in combination with the Ion Torrent Genexus Integrated Sequencer. **A:** Overall percentage of variants identified for each gene. **B:** Range of fusion and splice variants. **C:** Single-nucleotide variants (SNVs)/insertion and/or deletion variants. **D:** Copy number gain in cancer-associated genes detected in the study (genes with copy number loss are not shown). **E:** Confirmation by fluorescence *in situ* hybridization of *MET* copy number gain detected by OPA, demonstrating copy number gain validation. Chromosome 7 centromere (orange) and *MET* gene (green) are shown. Scale bar = 10 μm (**E**). Original magnification, ×100 (**E**).

using a non−fully automated NGS platform, which is estimated to be approximately 2 weeks.[46] In addition, associations between overall survival and cancer biomarker detection are also reported.[22] This approach has the potential to enable rapid molecular characterization of multiple patients simultaneously and offers significant benefits. Zheng et al[47] conducted a literature review to assess the diagnostic and economic value of NGS compared

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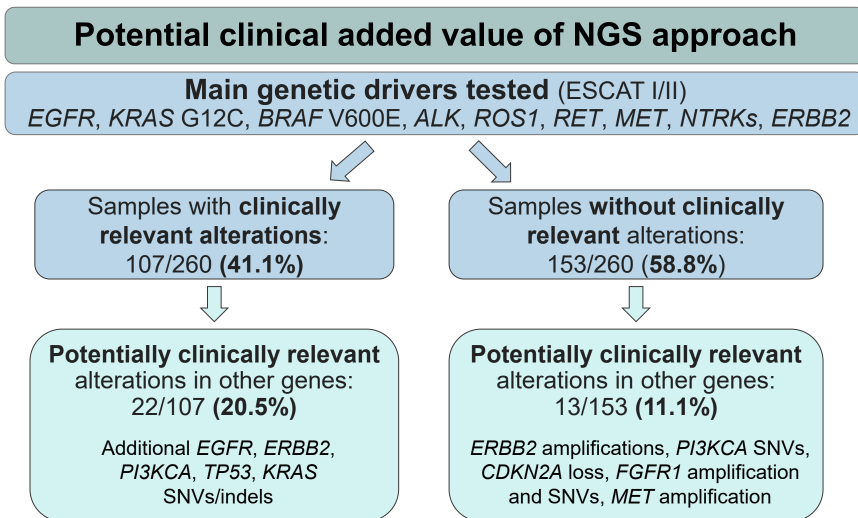

Figure 4 Prospective study: potential clinical added value of the next-generation sequencing (NGS) approach. **Left:** Frequency of non—small-cell lung cancer (NSCLC) samples that are positive for the main genetic drivers tested and also harbor potentially clinically relevant alterations. **Right:** Frequency of NSCLC samples that are negative for mutations in the main genetic drivers tested but positive for potentially clinically relevant alterations in other genes. ESCAT, European Society for Medical Oncology Scale for Clinical Actionability of Molecular Targets; indel, insertion and/or deletion variant; SNV, single-nucleotide variant.

with single-gene testing in the analysis of NSCLC biomarkers and showed that NGS is cost-effective. Moreover, Arriola et al[46] have shown that NGS can result in a greater proportion of patients receiving targeted therapy and gaining more years of life, while being cost neutral or cost saving. Another recent Italian study compared the total costs of these contrasting approaches, with single-gene techniques costing between $512 and $4066 and NGS between $539 and $3386. These calculations estimated savings of $32 to $1343 per patient analyzed using an NGS-based approach, depending on the number of biomarkers tested and the complexity of the methods (currency conversion from EUR to USD on November 7, 2024).[17] It is beyond the scope of this study to estimate the economic impact of using the proposed in-house NGS testing method compared with the previous molecular testing approach, mainly because of the limited period of use in each center. However, it can be noted that, at least in center B, the new approach reduced the number of staff units dedicated to molecular testing.

In addition to the reduced TAT, the in-house NGS testing approach showed a high reproducibility among centers, with 95.2% concordance among samples tested. In one case, partial concordance was observed because a single center detected an *EGFR* variant with a low allele fraction, which was then validated by real-time PCR. The discrepancy in this case can be explained by two main hypotheses. First, the center where the mutation was detected received the last set of slides, so these slides may be enriched with neoplastic cells compared with the sets received by the other two centers. Second, the center where the variant was detected is the only center that enriches the samples for NGS testing using a microscope-assisted microdissection approach, potentially increasing the proportion of tumor cells in the sample being analyzed and increasing the overall sensitivity of the test.

Several studies confirm the sensitivity of OPA in combination with the Genexus System in small sample sizes or low tumor cell content,[23,24] and even in liquid biopsies.[25,44] Interestingly, in this multicenter study, one center (center A) had only been using the Genexus Integrated Sequencer for 1 month when the project began, in contrast to the other centers, which had 7 to 8 months of experience. The limited experience with the instrument did not affect the results obtained, thereby highlighting the simplicity and robustness of OPA in combination with the Genexus System. Overall, adopting this combination in all centers brought several benefits. These included reducing the time to report molecular data (from 10—12 working days to 3—6 working days) and maintaining routine workloads even with an overall increase in the average number of cases tested per month (center A, 20%; center B, 29%; and center C, 25% increase per month), with no change in the number of personnel dedicated to tumor molecular profiling. In center B, this even led to a reduction in the number of dedicated personnel (from five to three). In addition, this NGS platform had a positive impact on NGS results from RNA by helping to reduce the RNA failure rate in samples from external sources. Gene fusion analysis is a critical aspect of precision medicine in lung cancer. However, it can be challenging to perform RNA-based sequencing on scant and/or low-quality diagnostic tissue specimens. Therefore, an NGS system that enables the detection of fusions with a low failure rate is crucial. This facilitates comprehensive molecular characterization and helps to identify NSCLC tumors that harbor *ALK*, *ROS1*, *RET*, and *NTRK1/2/3* gene rearrangements, which can benefit from treatment with TKIs.

Taken together, these results demonstrate the future benefits of performing rapid and easy-to-use NGS testing in-house to improve the accessibility and reproducibility of NGS in hospitals, contributing to the democratization of NGS, which is key to accelerating cancer treatment.

Another important finding of this study is the ability to detect not only all currently established biomarkers for

NSCLC, but also novel alterations in several genes that are potentially relevant to this tumor type.[5,6,48] Novel or uncommon *EGFR* mutations have been identified in NSCLC, some of which (eg, *EGFR* G598V) co-occur with other more common sensitizing *EGFR* mutations. This supports recent evidence obtained using NGS technologies showing that approximately 10% of tumors harbor compound *EGFR* mutations defined by the presence of dual or multiple distinct *EGFR* genetic alterations at baseline.[49] Moreover, alterations were identified in approximately 20% of samples that had mutations in established NSCLC biomarkers and may, therefore, be of interest as additional relevant genes. Among those genes, *TP53* is a negative predictor of response to first-line TKI treatment, including strategies against *EGFR*, *ALK*, and *ROS1* alterations.[36] *PI3KCA* alterations affect response to epidermal growth factor receptor (EGFR) TKIs.[33] Importantly, >10% of the samples identified as wild type for the main oncogenic drivers investigated (*EGFR*, *KRAS* G12C, *BRAF* V600E, *ALK*, *ROS1*, *RET*, *MET*, *NTRK*s, and *ERBB2*) had potentially clinically relevant alterations. Among those detected in the present series, *PI3KCA* alterations are not only potentially negative predictive markers for EGFR TKI treatment, but also novel potential targets,[33] as well as *FGFR1* amplifications and SNVs,[41] *ERBB2*,[31] and *MET*[42] amplifications, whereas loss of *CDKN2A* has been associated with immunotherapy response profiles.[38]

This raises an important point of discussion regarding the consistency of reporting NGS results. In this study, there were differences in reporting between the three centers. One center reported NGS data only for established NSCLC biomarkers and submitted additional NGS data on request, whereas the other two centers reported established biomarkers concurrently with relevant alterations in other genes. Standardizing the approach to reporting NGS data could significantly impact the identification of novel biomarkers for NSCLC and other tumors, which, in turn, would affect the development and implementation of targeted therapies and personalized medicine.

Conclusion

The in-house targeted NGS testing used in this study demonstrated high reproducibility, rapid TAT, and the ability to reliably identify known and novel mutations that could be therapeutic targets for future NSCLC management. This study confirms the importance of standardizing NGS guidelines to enable robust application across diverse laboratories, which could enable molecular diagnosis of NSCLC in the future.

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Disclosure Statement

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Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.jmoldx.2025.02.001*.

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Supplemental Figure 1 Assessment of the accuracy of *EGFR* p.G719A mutation detection. **A:** Detection of *EGFR* p.G719A mutation at an allele fraction of 3.5% in sample A5, analyzed by center C using the Oncomine Precision Assay on the Ion Torrent Genexus system. **B:** Screenshot from Integrative Genomics Viewer illustrating the nucleotide (Nuc.) substitution in *EGFR*. **C:** Real-time PCR analysis of sample A5 by center C confirmed the presence of the *EGFR* p.G719A mutation. CNV, copy number variant; ID, identifier; Indel, insertion and/or deletion variant; SNV, single-nucleotide variant.