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Original Article

Validation and Clinical Application of ONCOaccuPanel for Targeted Next-Generation Sequencing of Solid Tumors

Moonsik Kim[1], **Changseon Lee**[2], **Juyeon Hong**[2], **Juhee Kim**[3], **Ji Yun Jeong**[1], **Nora Jee-Young Park**[1], **Ji-Eun Kim**[1], **Ji Young Park**[1]

[1]*Department of Pathology, Kyungpook National University Chilgok Hospital, School of Medicine, Kyungpook National University, Daegu,* [2]*Bioinformatics Team, R&D Center, NGeneBio Inc., Seoul,* [3]*Diagnostics Development A Team, R&D Center, NGeneBio Inc., Seoul, Korea*

Purpose Targeted next-generation sequencing (NGS) is widely used for simultaneously detecting clinically informative genetic alterations in a single assay. Its application in clinical settings requires the validation of NGS gene panels. In this study, we aimed to validate a targeted hybridization capture-based DNA panel (ONCOaccuPanel) using the Illumina MiSeq sequencing platform. The panel allows the simultaneous detection of single-nucleotide variants (SNVs), insertions, deletions, and copy number changes of 323 genes and fusions of 17 genes in solid tumors.

Materials and Methods We used 16 formalin-fixed paraffin-embedded (FFPE) tumor samples with previously known genetic mutations and one reference material (HD827) for validation. Moreover, we sequenced an additional 117 FFPE tumor samples to demonstrate the clinical utility of this panel.

Results Validation revealed a 100% positive percentage agreement and positive predictive value for the detection of SNVs, insertions, deletions, copy number changes, fusion genes, and microsatellite instability–high types. We observed high levels of reproducibility and repeatability (R^2 correlation coefficients=0.96-0.98). In the limit of detection assessment, we identified all clinically relevant genes with allele frequencies > 3%. Furthermore, the clinical application of ONCOaccuPanel using 117 FFPE samples demonstrated robust detection of oncogenic alterations. Oncogenic alterations and targetable genetic alterations were detected in 98.2% and 27.4% cases, respectively.

Conclusion ONCOaccuPanel demonstrated high analytical sensitivity, reproducibility, and repeatability and is feasible for the detection of clinically relevant mutations in clinical settings.

Key words High-throughput nucleotide sequencing, DNA mutational analysis, Genetic testing, Validation, Neoplasms

Introduction

Next-generation sequencing (NGS) is a widely employed sequencing platform in the era of precision medicine [1]. Conventional molecular technologies, such as Sanger sequencing and polymerase chain reaction (PCR), can cover only hotspots of limited number of genes. In contrast, NGS technology can simultaneously detect hundreds of genetic alterations [2,3]. Tumors with an identical histologic diagnosis can comprise diverse types of driver gene mutations. For example, oncogenic mutations occurring in lung adenocarcinoma include the *KRAS*, *EGFR*, *ALK*, *ROS1*, *RET*, *MET*, *NTRK1-3*, *ERBB2*, *BRAF*, *MAP2K1*, and *NRG* [4-6]. In addition, multiple co-occurring genetic alterations affect tumor heterogeneity and significantly contribute to its biologic behavior [7,8]. Thus, NGS is key to appropriate patient diagnosis and establishment of a treatment plan.

Currently, NGS approaches can be broadly categorized into targeted panel sequencing, whole-exome sequencing, and whole-genome sequencing [9]. Whole-exome and whole-genome sequencing approaches are widely used for research purposes; however, targeted panel sequencing is feasible for the detection of clinically relevant genetic alterations [10]. The NGS workflow involves a complex process comprising multiple steps that require optimization, familiarization, and validation [11-14]. Nonetheless, there are limited standardized validation guidelines for NGS because of the diverse types of tumor samples, available sequencing platforms, and data analysis algorithms. However, several groups have provided the validation guidelines for the NGS process [10,15]. The validation of the NGS process is critical for patient care because every step of NGS harbors potential sources of errors that can harm the patients.

In this study, we aimed to validate and demonstrate the analytical performance of ONCOaccuPanel (NGeneBio, Seoul, Korea) on the Illumina MiSeq platform. ONCOaccu-

Correspondence: Ji Young Park
Department of Pathology, Kyungpook National University Chilgok Hospital, School of Medicine, Kyungpook National University, 807 Hoguk-ro, Buk-gu, Daegu 41404, Korea
Tel: 82-53-200-3408 Fax: 82-53-200-3399 E-mail: jyparkmd@knu.ac.kr
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Panel is a hybridization capture-based DNA panel that can detect somatic mutations and copy number alterations of 323 key cancer genes and fusions of 17 genes in solid tumors. Using numerous formalin-fixed paraffin-embedded (FFPE) samples of different tumor types, we further intended to demonstrate the clinical performance of ONCOaccuPanel. This panel is currently employed as a molecular diagnostic tool in several hospitals across Korea; thus, it is necessary to assess its clinical performance.

Materials and Methods

1. Study samples

We used 16 patient-derived FFPE samples and one reference sample (OncoSpan gDNA, cat No. HD827, Horizon Discovery, Waterbeach, UK), which comprised a wide range of mutations (single-nucleotide variants [SNVs] and insertions and deletions [Indels]) with known variant allele frequencies, for validation. The FFPE samples included samples of lung cancer (n=7), ovarian cancer (n=2), endometrial cancer (n=2), head and neck cancer (n=1), skin cancer (n=1), thyroid cancer (n=1), and malignancy of an unknown origin (n=2) (S1 Table). The tumor specimens and reference sample consisted of known genetic alterations detected by alternative molecular methods, including PCR, fluorescence *in situ* hybridization, and targeted NGS.

Furthermore, we investigated the analytical performance of ONCOaccuPanel in daily practice using 117 patient-derived samples (116 FFPE samples and one cytology sample). The included patient-derived samples were of 11 different tumor types—ovarian cancer (n=44), lung cancer (n=27), endometrial cancer (n=19), breast cancer (n=8), malignancy of an unknown origin (n=7), bone and soft tissue tumors (n=3), skin cancer (n=2), head and neck cancer (n=2), uterine cervix cancer (n=2), thyroid cancer (n=2), and stomach cancer (n=1). All FFPE samples displayed tumor cellularity of ≥ 10%. Two pathologists experienced in surgical pathology (Kim M and Jeong JY) reviewed the available stained slides and determined tumor cellularity. All samples were obtained from the Department of Pathology, Kyungpook National University Chilgok Hospital. S2 Table presents detailed information on the patient-derived tumor samples.

2. DNA extraction

The FFPE slices (5-μm-thick) were deparaffinized and rehydrated with xylene and alcohol solutions. We performed DNA extraction and purification using the Maxwell FFPE Plus DNA Kit (Promega, Madison, WI) according to the manufacturer's instructions. For the cytology specimen, DNA extraction was performed using the QIAamp DSP DNA Mini Kit (Qiagen, Hilden, Germany). We measured the DNA quantity by fluorometric quantification using a Quantus Fluorometer with a QuantiFluor dsDNA system (Promega). We evaluated the DNA integrity number (DIN) using an Agilent 4200 TapeStation (Agilent Technology, Santa Clara, CA).

3. Library preparation and next-generation sequencing

For sequencing the library preparation, genomic DNA was sheared using a Qsonica sonicator (Qsonica, Newtown, CT). We measured the size of the fragmented DNA using the Agilent 4200 TapeStation and High Sensitivity D1000 ScreenTape with High Sensitivity D1000 Reagents. Library construction was performed using ONCOaccuPanel. ONCOaccuPanel DNA probes were designed for the targeted sequencing of all exons and selected introns of 225 genes and partial exons of 98 genes (a total of 323 genes). S3 Fig. presents detailed information on the gene panel. It included 3,722 coding exons, with a total target size of 1.15 Mb. We performed end repair, A-tailing, ligation, and PCR amplification according to the manufacturer's instructions. The size of the pooled library was measured using the Agilent 4200 TapeStation and High Sensitivity D1000 ScreenTape with High Sensitivity D1000 Reagents. Library pooling, hybridization, capture, and post-capture PCR enrichment were performed according to the manufacturer's instructions. To assess the quality and quantity of the constructed library, we used the Agilent 4200 TapeStation and High Sensitivity D1000 ScreenTape with High Sensitivity D1000 Reagents to measure the library size. The pooled libraries were sequenced on a MiSeq sequencer (Illumina, San Diego, CA).

4. Determination of coverage requirements and target region coverage

Theoretically, a true mutation with ≥ 10% frequency can be detected with 100× coverage and 98% confidence (power) (α=0.05). Thus, we were likely to capture the majority of true-positive (TP) variants with ≥ 10% frequency at 100× coverage (95% confidence interval of variant allele frequency [VAF], 4.9 to 17.6). To achieve ≥ 100× coverage for ≥ 98% of the targeted exons, the samples were sequenced to ≥ 200× mean coverage. To validate this hypothesis, we sequenced 20 clinical samples (provided by NGeneBio) using the Illumina MiSeq platform. Most genes (95.1%, 307/323) displayed > 100× coverage. The minimum coverage of the 14 essential genes (*ALK*, *APC*, *BRAF*, *BRCA1*, *BRCA2*, *EGFR*, *ERBB2*, *IDH1*, *IDH2*, *KIT*, *KRAS*, *MET*, *PIK3CA*, and *TP53*) that should be included in NGS tests in Korea is 169×. Among the 3,722 total exons, 97 displayed low coverage, i.e., < 100×. Among the 14 essential genes, exon 1 of *BRAF*, *ERBB2*, and *IDH2* revealed high GC contents (77.13%, 76.52%, and 76.27%, respectively), with a low coverage, i.e., < 100×. S4 Table summarizes the

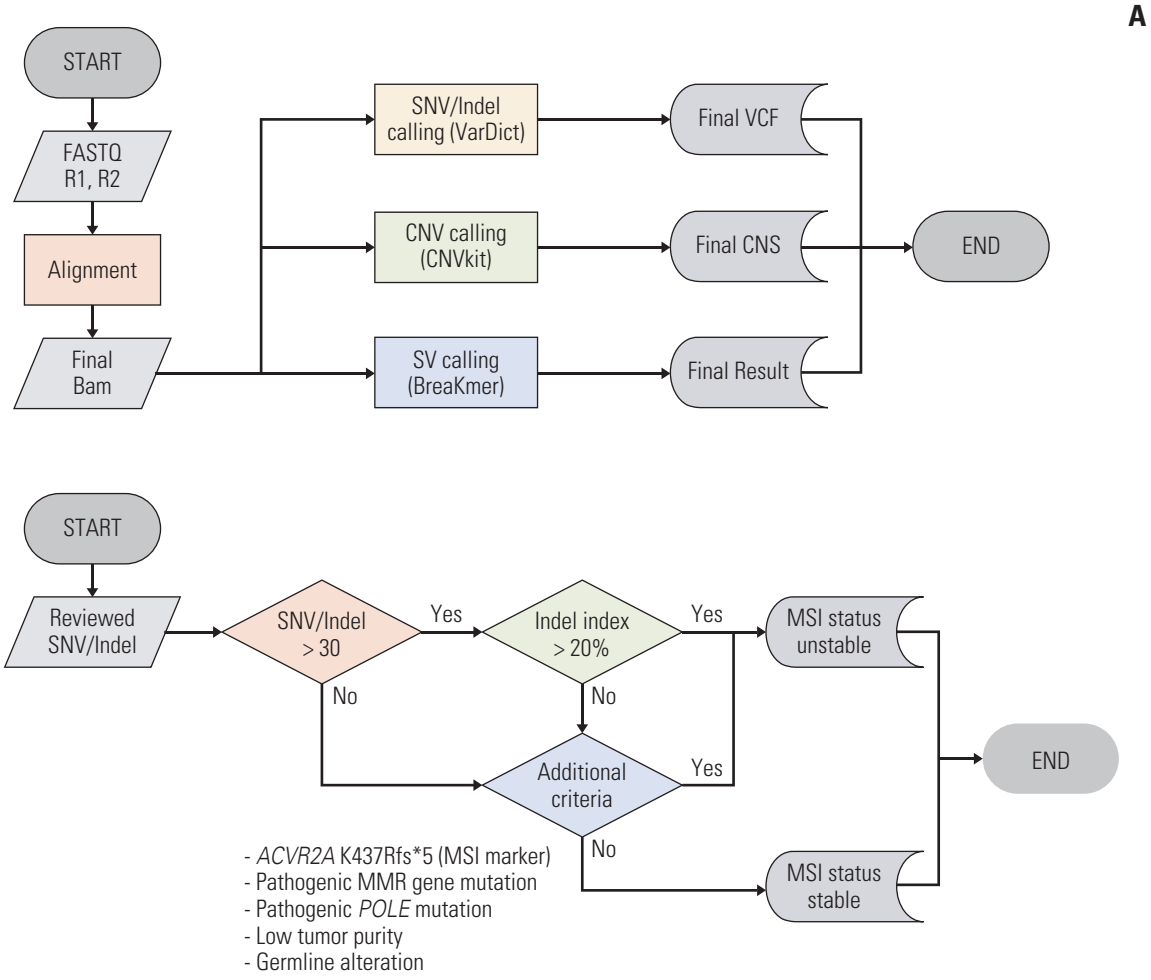

Fig. 1. Bioinformatics pipeline analysis process of ONCOaccuPanel: overall process (A) and SNV and Indel calling process (B). CNV, copy number variation; Indel, insertion and deletion; MAF, Mutation Annotation Format; MMR, mismatch repair; MSI, microsatellite instability; POLE, polymerase ε; SNV, single-nucleotide variant; SV, structural variation; VCF, Variant Call Format. *(Continued to the next page)*

coverage information of all genes, GC contents, and the list of exons with < 100× coverage.

5. Bioinformatic pipeline

Fig. 1 depicts a bioinformatics pipeline flow chart. We assessed the quality control of the FASTQ files generated by ONCOaccuPanel using FastQC (ver. 0.11.3). Subsequently, we used the Burrows-Wheeler Aligner to align reads to the human reference genome (GRCh37;hg19). Consequently, MarkDuplicates (Picard) were used to identify and tag the duplicate reads in a BAM file. We realigned the Indel mutations using the Genome Analysis Tool Kit (GATK) (ver. 4.2.6.1) IndelRealigner. The base pair quality scores were calculated using the GATK CountCovariates and TableRecalibration functions.

We used VarDict (ver. 1.6) for SNV and Indel calling. To reduce the false-positive calls, we filtered the following variants: (1) total read depth < 30, mutated read counts < 3, and variants with a VAF < 3% and (2) minor allele frequency > 1% in the Genome Aggregation Database, Single Nucleotide Polymorphism Database, Exome Aggregation Consortium, and Korean Reference Genome Database. All genes with blacklist variants and panel of normal were excluded from the analysis. However, well-known and clinically significant genetic alterations which are validated predictive biomarkers of drug response or have prognostic implication in a specific cancer subtype, such as *EGFR* exon 19 deletion or *KRAS* G12C mutation in lung cancer (so called whitelisted variant), were not filtered and were reviewed manually using the Integrative Genomics Viewer, even though they did not pass the filtering criteria.

For copy number variation (CNV) analysis, we used the

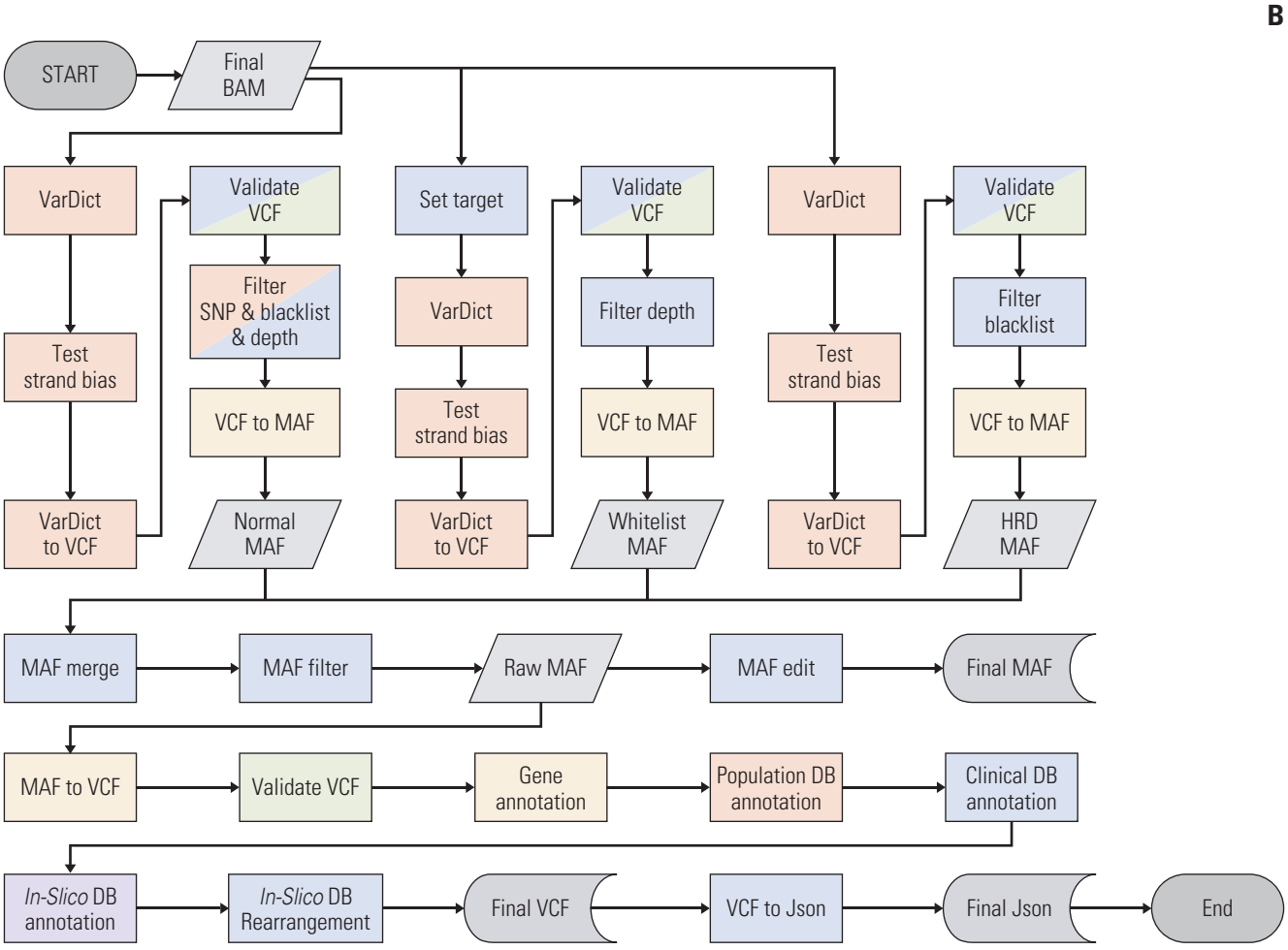

Fig. 1. *(Continued from the previous page)*

CNVkit to estimate the copy ratios of the tumor to pooled normal samples. The copy number was measured using the following equation:

Estimated copy number=([200×reported fold change]–2×[100–tumor purity (%)])/tumor purity (%).

Genes with an estimated copy number ≥ 5 were reported as an amplification, whereas those with an estimated copy number ≤ 0 were reported as deletions.

We detected gene fusion using BreaKmer (ver. 0.0.2). Split (chimeric) reads with a read count < 2 were considered false positives and filtered.

The microsatellite instability–high (MSI-H) type was defined as the total number of somatic mutation (SNV/Indel) > 30 and Indel index (Indel mutation to total mutation ratio) > 20%. The MSI-H cutoff criteria was similar to that suggested in the previous study [16]. However, due to the difference in the numbers of genes included in the panel, tumor types examined, and presence of low tumor cellularity samples in clinical practice which can influence the detection of SNV/Indel, the cutoff for MSI-H was altered (SNV/Indel: from > 40 to > 30, Indel index: from 9% to 20%). Moreover, *ACVR2A* K43Rfs*5 mutations or oncogenic DNA polymerase epsilon catalytic subunit gene alterations, including P286R, V411L, F367C, and S279Y, were considered MSI-H types, as previously described [16].

Clinical actionability of genetic alterations was determined based on the following reference databases: Cancer Genome Interpreter (CGI), Clinical Interpretation of Variants in Cancer (CIViC), Catalogue Of Somatic Mutations In Cancer (COSMIC), Clinically relevant Variation (ClinVar), and MSK's Precision Oncology Knowledge Base (OncoKB).

6. MSI testing

PCR using five National Cancer Institute (NCI) markers

Table 1. Predictive value of OncoAccupanel

	No.	TP	FP	TN	FN	PPV (%)	PPA (%)
SNV	28	28	0	0	0	100	100
Indel	10	10	0	0	0	100	100
CNV	5	5	0	0	0	100	100
SV	2	2	0	0	0	100	100
MSI-high	3	3	0	0	0	100	100

CNV, copy number variation; FN, false-negative; FP, false-positive; Indel, insertions and deletion; MSI, microsatellite instability; PPA, positive percentage agreement; PPV, positive predictive value; SNV, single-nucleotide variant; SV, structural variation; TN, true negative; TP, true-positive.

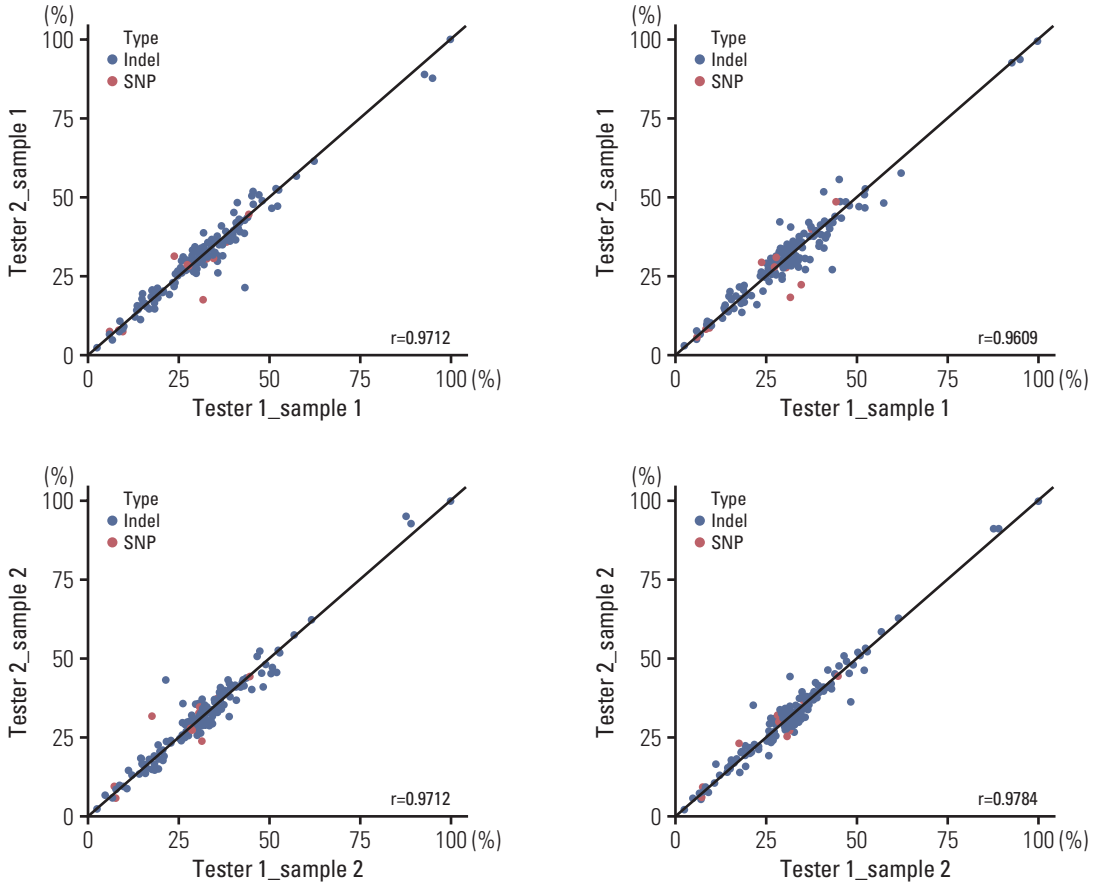

Fig. 2. Repeatability test of ONCOaccuPanel. Percentages in the vertical axis and horizontal axis denote the variant of allele frequency (VAF). Indel, insertion and deletion; SNP, single nucleotide polymorphism.

(BAT-26, BAT-25, D5S346, D17S250, and S2S123) was performed to determine the MSI status of the tumors. Representative tumor and matched normal tissues were used for MSI testing. A DNA autosequencer (ABI 3731 Genetic Analyzer, Thermo Fisher Scientific, Waltham, MA) was used to analyze the PCR products. According to the revised Bethesda Guidelines [17], tumors with at least two markers with unstable peaks were classified as MSI-H, tumors with one unstable marker were defined as microsatellite instability–low (MSI-L), and tumors with no unstable markers were designated as microsatellite stable (MSS). However, recent guidelines have demonstrated that MSI-L should be included with MSS [18]. Thus, in this study, the status of microsatellites was classified into two classes: MSI-H and MSS.

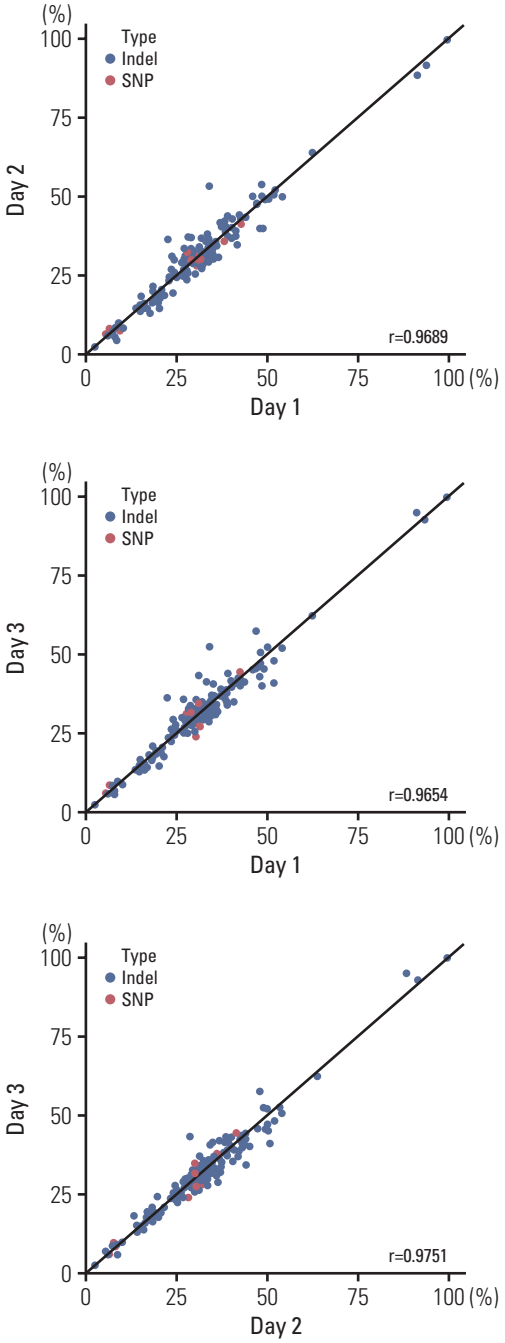

Fig. 3. Reproducibility test of ONCOaccuPanel. Percentages in the vertical axis and horizontal axis denote the variant of allele frequency (VAF). Indel, insertion and deletion; SNP, single nucleotide polymorphism.

7. Mismatch repair protein immunohistochemistry

Mismatch repair (MMR) protein immunohistochemistry (IHC) was performed using primary monoclonal antibodies against MLH1 (clone 760-5091, Ventana, Tucson, AZ), MSH2 (clone 760-5093, Ventana), MSH6 (clone 790-4455, Ventana), and PMS2 (clone 288M-16, Cell Marque, Rocklin, CA) following the automated standard protocol. Mismatch repair deficient status was defined as complete absence of nuclear staining within the tumor with retained positive nuclear staining in non-neoplastic cells.

8. PCR analysis for *EGFR*, *BRAF*^{V600E} mutation and *ROS1* fusion

To detect *EGFR* and *BRAF*^{V600E} mutations, the PANAMutyper EGFR Kit (Panagene) and the PNA Clamp BRAF Mutation Detection Kit (Panagene) were used according to the manufacturer's instructions, respectively. Additionally, we performed real-time PCR using the ROS1 Gene Fusions Detection Kit (AmoyDx, Xiamen, China) to detect *ROS1* rearrangements.

9. Statistical analyses

The positive percentage agreement (PPA) was calculated as TP/TP+false-negative, whereas the positive predictive value (PPV) was calculated as TP/TP+false-positive. We evaluated the reproducibility using Pearson correlation coefficient. All statistical analyses were performed using IBM SPSS Statistics ver. 23 (IBM Corp., Armonk, NY).

Results

1. Validation of ONCOaccuPanel

(1) Assessment of panel accuracy

We used 16 patient-derived FFPE samples and one reference material (HD827) for the accuracy test. These FFPE samples included previously confirmed genetic mutations (16 SNV, four Indels, five CNV, two structural variations [SVs], and three MSI-H types). S1 Table presents the detailed quality metrics for the FFPE samples. Briefly, all samples, except one, had an average on-target coverage of at least 250×. The average on-target rate was 65.8% (60.0%-73.0%). For the reference sample, we only included verified mutations with a known VAF (SNV 12, Inel 6) (S5 Table). For both the FFPE and reference material, the PPA and PPV were 100% for SNVs, Indels, CNV, SV, and MSI-H types (Table 1).

(2) Reproducibility and repeatability

To demonstrate the precision of ONCOaccuPanel, we performed reproducibility and repeatability tests using the reference material HD827. The SNVs and Indels were separately evaluated. The repeatability tests were performed with two replicates, each by two technicians in a single run. The reproducibility tests were performed by a single technician in three different runs. The repeatability tests showed an R^2 cor-

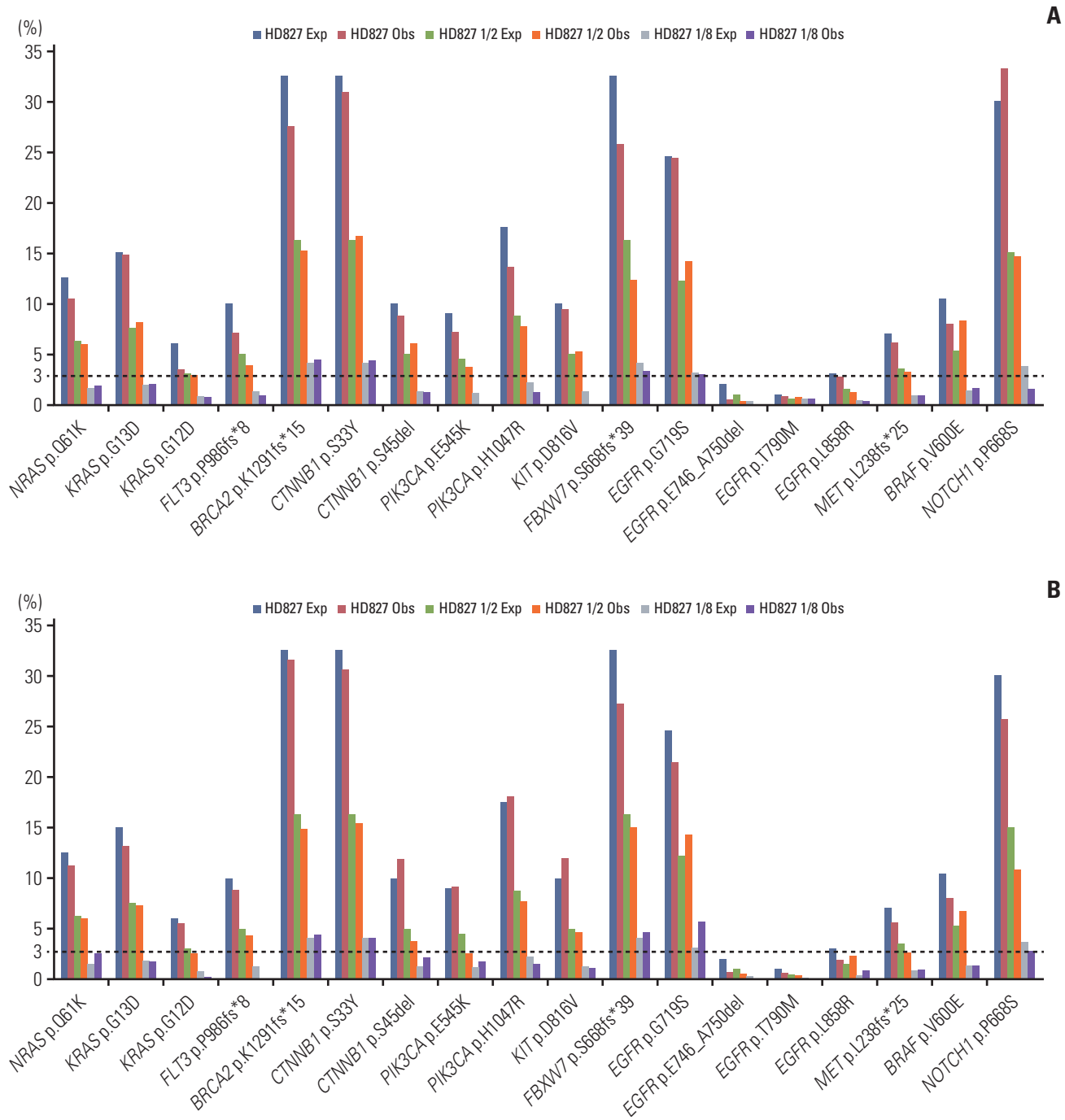

Fig. 4. Estimating the limit of detection of OncoAccupanel; first (A) and second (B) test. Percentages in the vertical axis denote the variant of allele frequency (VAF). Exp, expected VAF; Obs, observed VAF.

relation coefficient of 0.96-0.98 (Fig. 2). The reproducibility tests showed an R^2 correlation coefficient of 0.97-0.98 (Fig. 3).

(3) Limits of detection

We evaluated the limit of detection (LOD) by sequentially diluting the reference material (1/1, 1/2, and 1/8). The LOD estimation test was performed twice in two separate runs. In both runs, all clinically relevant genes were consistently detected with a VAF of > 3% (Fig. 4). With the expected VAF of 0.5%-3%, we detected 21 of 24 (87.5%) SNVs and 11 of 12 (91.7%) Indels (Table 2).

Table 2. Estimating the limit of detection of OncoAccupanel

Gene	Amino acid change	Dilution	Expected VAF (%)	First	Second
				Observed VAF (%)	Observed VAF (%)
NRAS	p.Q61K	1	12.50	10.50	11.20
		1/2	6.30	6.00	6.10
		1/8	1.60	1.90	2.60
KRAS	p.G13D	1	15.00	14.80	13.10
		1/2	7.50	8.10	7.30
		1/8	1.90	2.00	1.80
KRAS	p.G12D	1	6.00	3.50	5.60
		1/2	3.00	3.00	2.50
		1/8	0.80	0.70	0.30
FLT3	p.P986fs*>8	1	10.00	7.10	8.90
		1/2	5.00	3.90	4.40
		1/8	1.30	0.80	-
BRCA2	p.K1691fs*15	1	32.50	27.50	31.60
		1/2	16.30	15.20	14.90
		1/8	4.10	4.40	4.40
CTNNB1	p.S33Y	1	32.50	30.90	30.50
		1/2	16.30	16.70	15.50
		1/8	4.10	4.40	4.10
CTNNB1	p.S45del	1	10.00	8.80	11.90
		1/2	5.00	6.00	3.80
		1/8	1.30	1.20	2.10
PIK3CA	p.E545K	1	9.00	7.20	9.20
		1/2	4.50	3.60	2.70
		1/8	1.10	-	1.80
PIK3CA	p.H1047R	1	17.50	13.70	18.00
		1/2	8.80	7.70	7.70
		1/8	2.20	1.20	1.50
KIT	p.D816V	1	10.00	9.40	12.00
		1/2	5.00	5.20	4.70
		1/8	1.30	-	1.10
FBXW7	p.S668fs*39	1	32.50	25.80	27.20
		1/2	16.30	12.30	15.00
		1/8	4.10	3.20	4.70
EGFR	p.G719S	1	24.50	24.30	21.40
		1/2	12.30	14.10	14.40
		1/8	3.10	3.00	5.70
EGFR	p.E746_A750del	1	2.00	0.50	0.70
		1/2	1.00	0.40	0.60
		1/8	0.30	-	-
EGFR	p.T790M	1	1.00	0.80	0.70
		1/2	0.50	0.70	0.40
		1/8	0.10	-	-
EGFR	p.L858R	1	3.00	2.80	1.90
		1/2	1.50	-	2.30
		1/8	0.40	0.30	0.80
MET	p.L238fs*25	1	7.00	6.10	5.60
		1/2	3.50	3.20	2.70
		1/8	0.90	0.80	1.00

(Continued to the next page)

Table 2. Continued

Gene	Amino acid change	Dilution	Expected VAF (%)	First Observed VAF (%)	Second Observed VAF (%)
BRAF	p.V600E	1	10.50	7.90	8.00
		1/2	5.30	8.30	6.70
		1/8	1.30	1.60	1.40
NOTCH1	p.P668S	1	30.00	33.20	25.70
		1/2	15.00	14.70	10.90
		1/8	3.80	1.50	2.80

VAF, variant allele frequency.

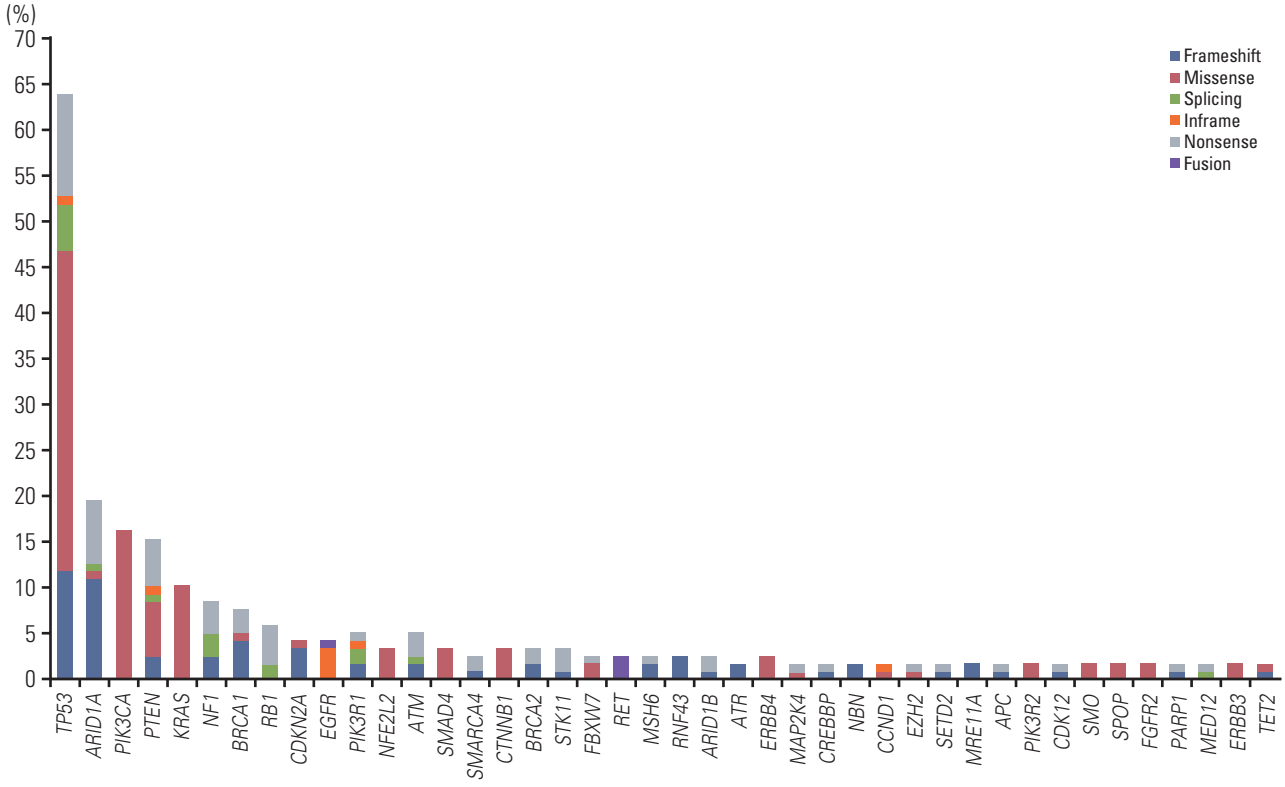

Fig. 5. Oncogenic mutations and fusions detected in 117 clinical samples.

2. Clinical performance

(1) Quality metrics

In the 117 clinical samples, the average total number of DNA reads was 10,500,399 (range, 4,741,992 to 15,574,808). Most samples (104/117, 88.9%) displayed an average on-target coverage of at least 250×, and all samples achieved an on-target coverage of > 100×. The average on-target rate was 64.6% (range, 48.7% to 72.5%). S2 Table presents detailed information regarding the quality metrics.

(2) Analytical performance in real-world samples

We evaluated the clinical utility of ONCOaccuPanel using clinical samples. According to the Association for Molecular Pathology/American Society of Clinical Oncology/College of American Pathologists variant categorization [19], we identified tier I (variants of strong clinical significance) or tier II (variants of potential clinical significance) variants in 115 of 117 cases (98.3%). Targetable genetic alterations, which are Food and Drug Administration–approved as predictive biomarkers of drug response, were observed in 33 of 117 cases (27.4%). Among all oncogenic mutations, the frequently

altered genes were *TP53* (75/117, 64.1%), *ARID1A* (23/117, 19.7%), *PIK3CA* (19/117, 16.2%), *PTEN* (18/117, 15.4%), *KRAS* (14/117, 12.0%), *NF1* (10/117, 8.5%), *BRCA1* (9/117, 7.7%), *RB1* (7/117, 6.0%), *ATM* (6/117, 5.2%), *PIK3R1* (6/117, 5.2%), *CDKN2A* (5/117, 4.3%), and other mutations with lower frequencies (Fig. 5, S2 Table). We identified oncogenic gene fusion in five cases (three *RET* fusions, one *ROS1* fusion, and one *EGFR* fusion). Among all *KRAS* mutations, *KRAS* G12C mutation was identified in five cases. Among the eight breast cancer cases, *PIK3CA* mutations were observed in three cases. All breast cancer cases were of the triple-negative subtype. We identified complex copy number alterations in seven of eight breast cancer cases (87.5%). The MSI-H type was observed in five cases (5/117, 4.3%). All MSI-H–type cancers were uterine endometrioid carcinomas. All oncogenic mutations in *BRCA1* and *BRCA2* were observed in ovarian cancer. We identified gene amplification in 45 cases, and *MYC* amplification was the most frequently detected (11/117, 9.4%). We identified gene deletion in 17 cases, including the deletion of *MTAP* (6/117, 5.1%), *CDKN2A* (5/117, 4.3%), and *CDKN2B* (5/117, 4.3%). We further validated the NGS results of MSI status and other genetic alterations by PCR and IHC (S6 Table). For all five MSI-H cases, both mismatch repair protein IHC and MSI PCR testing were performed. The concordance ratio was 100% for IHC. However, one MSI-H endometrioid carcinoma with *MSH6* truncating mutation (*MSH6* N897Kfs*3) was MSS by PCR (concordance rate 80%, 4/5). For 112 MSS cases, PCR tests were performed in 14 cases and mismatch repair protein IHC was performed in 27 cases. The concordance ratio was 100% for both PCR and IHC. For three *EGFR*-mutant lung cancers, *EGFR* mutations were detected in two cases by real-time PCR. One *EGFR* wild-type by PCR test was proved to be *EGFR* exon 20 insertion mutation by NGS, of which the probe was not included in the commercially available kit. Furthermore, we successfully validated other genes not discussed here, which are shown in S6 Table.

(3) Interesting cases

NGS detected *TPR-ROS1* fusion in a case of lung adenocarcinoma, which was not detected by a previous *ROS1* real-time PCR test. We hypothesized that it could function as an oncogenic driver owing to the retained tyrosine kinase domain of ROS1. ROS1 IHC revealed diffuse membranous positivity. Another case of lung adenocarcinoma harbored *EGFR* N771dup mutation, which is reportedly associated with the resistance to gefitinib and erlotinib. Considering the absence of a commercially available kit for the detection of *EGFR* exon 20 insertion mutations, the results of previous *EGFR* real-time PCR tests indicated wild-type *EGFR*.

Discussion

Recent advances in molecular genetics have led to the refined classification of tumors based on pathognomonic genetic alterations. The genomic profiling of tumors is essential for optimal treatment selection [20,21]. NGS can simultaneously identify the genetic alterations of hundreds of relevant genes in a single assay [22]. The NGS panel should be validated before its clinical application. In this study, we validated the analytical sensitivity, precision, and LOD of ONCOaccuPanel using patient-derived FFPE tumor samples and a reference sample, HD827. Moreover, we investigated its performance in real-world samples.

During validation, ONCOaccuPanel demonstrated excellent PPA (100.0%) and PPV (100.0%) for SNVs, Indels, CNVs, SVs, and MSI-H types. The reproducibility and repeatability tests demonstrated R^2 values of 0.96-0.98. During the LOD test, we identified all clinically relevant genes with an expected VAF of > 3%, and the quality metrics for the 16 FFPE samples revealed that the target coverage for all samples was > 250× except one. This finding satisfied the recommended sequencing depth of 250× and allele frequency of 5% as the cutoff for variants [23]. One sample with < 250× on-target coverage demonstrated a DIN of < 3 (2.1).

Quality metrics using additional 117 tumor samples demonstrated an average on-target coverage of 328×. Thirteen cases revealed an on-target coverage of < 250×. The sample quality, sample input, GC content, and other factors might have contributed to the low coverage of these samples [10]. However, all FFPE and cytology samples achieved an on-target coverage > 100×, thus suggesting that we could successfully detect genetic alterations with ≥ 10% VAF. This finding is noteworthy because most solid tumor specimens available for the NGS tests include FFPE samples.

We identified a wide range of clinically relevant genes, of which *TP53* was the most frequent gene. Considering the high proportion of ovarian, endometrial, and uterine cervical cancer samples, we frequently identified *ARID1A*, *PTEN*, *KRAS*, and *PIK3CA* mutations [24-27]. We identified *EGFR* mutations in only three of the 28 lung cancer cases. This is because most NGS performed in lung cancer comprised wild-type *EGFR* on previous PCR tests. Likewise, lung cancer cases were enriched with a diverse type of rare mutations, such as *TPR-ROS1* fusion, *EGFR-RAD51* fusion, *EGFR* exon 20 insertion mutation, *MET* exon 14 skipping mutation, *ERBB2* mutation, and *RET* fusion. Despite limited reports of *TPR-ROS1* fusion in lung cancer [28], its pathogenicity has been reported in other types of malignancies [29]. In addition, ROS1 IHC demonstrated diffuse cytoplasmic positivity, further supporting the oncogenic role in this case. All breast cancer cases included in this study were of the triple-negative

subtype. Consistent with previous studies, *PIK3CA* was the most frequently detected oncogenic alteration, thus providing an indication for targeted therapy for breast cancer [30]. Notably, we identified complex copy number alterations in most breast cancer cases (S2 Table). This may reflect an underlying homologous recombination deficiency [31]. However, researchers should perform the loss of heterozygosity test or homologous recombination deficiency scoring test to confirm this hypothesis [32]. We also successfully validated the NGS results with conventional diagnostic methods (PCR and IHC), although relatively small number of samples were used for the validation. One MSI-H endometrioid carcinoma case (MSI-H by NGS and deficient mismatch repair by IHC) harboring *MSH6* truncating mutation was MSS by MSI PCR. Although PCR testing is regarded as the gold standard to detect MSI-H tumors [33], it is well known that MSI-H tumors caused by defective *MSH6* gene can be MSS by PCR analysis [34,35]. We thus concluded that MSS by MSI PCR testing in this case was a false-negative result based on the results of the NGS test (more than 30 SNVs/Indels and Indel index > 20%) and MMR protein IHC results (loss of nuclear positivity of tumor cells on MSH6 immunostaining).

This study has some limitations. We did not assess the tumor mutational burden (TMB), which can elucidate immune checkpoint inhibitor treatment [36]. ONCOaccuPanel offers TMB values; however, we were unable to compare the TMB results with those of the reference standard method. Moreover, we used relatively fewer FFPE samples and reference materials during validation. In addition, we did not include diverse types of samples; most samples were FFPE samples, and we included only one cytological specimen. However, we could demonstrate good quality metrics with good clinical sensitivity to detect the oncogenic alterations using additional 117 samples in clinical settings. Furthermore, all subsequent NGS analyses in a clinical practice setting using cytology specimens (not included in this study) demonstrated good quality metrics with an on-target coverage > 250×. In this study, gene fusions were detected by ONCOaccuPanel, DNA-based panel, including some rare types of fusions. However, the detection of gene fusions by the DNA-based panel can be influenced by several factors including the size of intronic regions with repetitive sequences. We recently experienced a lung adenocarcinoma case with *CD74-ROS1* fusion that was filtered out during annotation due to the similarity of the sequence between *CD74* and the intergenic region (data not included). *ROS1* gene fusion was previously detected by real-time PCR. We were able to detect *CD74-ROS1* fusion by manual review of the case using the Integrative Genomics Viewer. Furthermore, combined DNA and RNA sequencing can be optimal in detecting a wide range of fusions with better sensitivity, including some rare gene fusions, such as *NTRK* fusion [37].

In summary, we validated and investigated the clinical utility of ONCOaccuPanel, a targeted NGS panel designed to detect clinically relevant genes. This NGS panel is feasible for the detection of oncogenic alterations in clinical practice. ONCOaccuPanel is widely employed in several major hospitals in Korea; therefore, this study will supposedly contribute to patient diagnosis and treatment with the relevant molecular classification of tumors.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

The study was conducted in accordance with the guidelines of the Declaration of Helsinki and was approved by the Daegu Joint Institutional Review Board (No. DGIRB 2021-12-004-001). The requirement for written informed consent from the patients was waived because of the retrospective study design.

Author Contributions

Conceived and designed the analysis: Kim M, Park JY.
Collected the data: Jeong JY, Park Nora JY, Kim JE.
Contributed data or analysis tools: Lee C, Hong J, Kim J.
Performed the analysis: Lee C, Hong J, Kim J, Kim JE.
Wrote the paper: Kim M, Jeong JY, Park Nora JY.

ORCID iDs

Moonsik Kim : https://orcid.org/0000-0002-5804-8790
Ji Young Park : https://orcid.org/0000-0002-7571-1064

Conflicts of Interest

Conflict of interest relevant to this article was not reported.

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