Comprehensive genomic profiling of Taiwanese triple-negative breast cancer samples with medium- and large-sized sequencing panels: A comparative study implicating treatment allocations

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Abstract. Comprehensive genomic profiling (CGP) is a molecular diagnostic tool with increasing use in cancer research and treatment. There are several commercialized CGP assays with variable targeted genes, while the differences between medium- and large-sized panels in refining genomic profiling strategies, optimizing resource allocation and enhancing clinical applications require evaluation. In the present study, patients with triple-negative breast cancer (TNBC) from the Veterans General Hospital TAipei-Yung-Ling foundation sinO-canceR study were initially assayed using a medium-sized CGP panel (Oncomine comprehensive panel), and the remaining nucleic acid specimens were re-sequenced with a large-sized panel (TruSight Oncology 500). The molecular profiling between the two sequencing panels was compared. A total of 108 breast cancer samples were successfully assayed using both platforms and 272 variants were reported at least once by either type of CGP. The reported variants were among actionable genes (*AKT1*, *BRCA1/2*, *PALB2*, *ERBB2*, *PIK3CA* and *PTEN*), which can be acted upon or have clinical relevance for therapeutic intervention and *TP53*. The concordance rate between the medium- and large-sized panels was 34.6%, which was enhanced to 58.9% after excluding polymorphisms, out-of-targeted region variants and those with low variant allele frequency (<10%), with variants of *TP53*, *ERBB2* and *PTEN* being mostly enhanced. A majority of discordance came from TruSight Oncology 500-detected only variants, especially *BRCA1*, *BRCA2* and *PALB2*. In conclusion, the results indicated that only one-third of actionable mutations could be detected consistently between the medium- and large-sized CGP panels using the default analytical pipelines, while extensive bioinformatics analyses improved concordance substantially. The large-sized panel detected more variants, thereby enhancing clinical actionability. With more therapeutic targets revealed in the future, CGP may be particularly impactful in refining strategies for TNBC management.

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Introduction

Comprehensive genomic profiling (CGP) is a molecular diagnostic tool, based on next-generation sequencing (NGS) technology, used in the field of cancer research and treatment. CGP identifies various genetic alterations and mutations within the tumor (1). Results from CGP guide oncologists to make decisions about treatment options and select targeted therapies tailored to the specific genetic alterations present within the tumor. CGP examines a broad panel of genes, detecting a wide range of genomic alterations. This includes

point mutations (such as *PIK3CA* p.E453Q and c.1357G>C), insertions/deletions (indels; such as *BRCA2* p.E1571Gfs*3 and c.4712_4713delAG), copy number variations (CNVs; such as *ERBB2* amplification, copy number:4) and gene fusions (such as *TBL1XR1*/*PIK3CA* fusion). It can detect multiple genetic alterations in a single test, reducing the need for multiple individual tests. CGP is an application of NGS that specifically focuses on a detailed analysis of the genetic profile of patients, and can provide a broad overview of the genomic alterations in a tumor. Consequently, precision, efficiency and therapeutic guidance are potential advantages of CGP, which has been advocated for advanced-stage cancers (2-5). A large-sized panel can provide more opportunities for matching patients to targeted therapies or for increased mutation detection in clinical trials, while the use of CGP is limited by its high cost, data complexity and increased turnaround time.

Triple-negative breast cancer (TNBC) is a specific subtype of breast cancer characterized by the absence of three deterministic receptors commonly found in other breast cancers: Estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). TNBC cells lack the expression of these three receptors and do not rely on them for growth. TNBC accounts for 10-20% of all breast cancer cases world (6), and is more commonly diagnosed in younger (<40 years old) women, African-American women and those with a family history of breast cancer (7). TNBC is known for its aggressive behavior and tends to grow and spread quickly, while it is also associated with a higher risk of recurrence and metastasis (8), and ~40% of people with stage I to stage III TNBC will exhibit tumor recurrence after standard treatment (9). Treatment options for TNBC often involve chemotherapy, as endocrine therapies and anti-HER2 therapies that target ER, PR or HER2 are not effective due to the absence of these receptors (8). The prognosis for TNBC varies depending on factors such as the diagnosed stage and response to treatment (10). Due to its aggressiveness, there is an unmet need to identify actionable targets such as *AKT1*, *BRCA1/2*, *PALB2*, *ERBB2*, *PIK3CA* and *PTEN,* for patients with TNBC (11,12).

Research focusing on developing targeted therapies for TNBC continues to improve treatment outcomes and broaden therapeutic options (13,14). For example, biomarker-driven therapies are being applied with the advent of immune checkpoint inhibitors, poly-adenosine-diphosphate-ribose polymerase (PARP) inhibitors, selective PI3K and AKT inhibitors, as well as antibody-drug conjugates, which target immune-enriched, DNA repair-deficient, PI3K/AKT/mTOR-activated and surface antigen-overexpressed TNBC, respectively. However, most umbrella trials for advanced breast cancer are not TNBC-specific, while a recent review highlighted that large-sized panels could identify novel markers, emphasizing the potential of CGP to uncover actionable targets in TNBC (3).

Commercialized CGP assays may be beneficial; however, the size of the panel to be employed to deliver the most optimal coverage of actionable genes for TNBC remains to be explored. Some studies comparing distinct CGP platforms and resulting biomarkers have been conducted for gastric, head and neck squamous cell carcinoma, non-small cell lung cancer, ovarian and prostate cancer, but rarely for TNBC (15,16). In the present study, a subgroup of patients with TNBC from the Veterans General Hospital TAipei-Yung-Ling foundation sinO-canceR (VGH-TAYLOR) study was used (17). TNBC samples were initially assayed using a medium-sized CGP panel, and the remaining specimens of nucleic acid were re-sequenced with a large-sized GCP panel. The aim of the present study was to investigate whether a larger CGP panel offers clinically significant advantages in detecting actionable variants for TNBC management compared with a medium-sized panel.

Materials and methods

Study overview. The present study was conducted in two phases: The first phase involved prospective and retrospective tissue collection as part of the VGH-TAYLOR study (medium-sized panel). The second phase involved the reuse of biobanked samples from the VGH-TAYLOR study (large-sized panel). The study was approved by the Institutional Review Board of Taipei Veterans General Hospital (approval nos. 2021-01-007B and 2023-08-002B; Taipei, Taiwan). Participants provided written informed consent for both phases of the study.

The VGH-TAYLOR study was designed to examine the genetic profiling of different subtypes of breast cancer in Taiwan (17). The prospective study comprised diverse clinical scenarios of breast cancer: i) Group 1, planned to receive first-line surgery followed by adjuvant therapy (1A) or exhibited early relapse within 3 years of diagnosis (1B); ii) group 2, planned to receive first-line neoadjuvant therapy followed by surgery; and iii) group 3, exhibited *de novo* stage IV (3-I) or recurrence beyond 3 years of diagnosis (3-II).

In addition, a retrospective cohort with biobanked samples from recurrent/metastatic breast cancers or patients with non-pathological complete response following neoadjuvant therapy was also included (18-21).

Inclusion criteria were a diagnosis of TNBC from the VGH-TAYLOR study, availability of sufficient biobanked nucleic acid and willingness to sign informed consent. Patients without residual samples and those unwilling to participate in the retesting program were excluded. A total of 120 female patients were screened, with 108 cases included for comprehensive genomic profiling. The age range was 27 to 84 years, with a mean ± SD age of 56.1±12.8 years. All enrolled subjects were treated according to contemporary guidelines and underwent regular follow-up (22,23). Regarding immunohistochemistry (IHC) testing, ER and PR were scored by percentage of nuclear labeling (0-100%). HER2 expression was scored using a 0 to 3+ membrane staining intensity score (24). Hormone receptor positivity was defined by either ER or PR with ≥1% of tumor cells exhibiting nuclear staining while HR-negative breast cancer was defined by the absence of ER and PR on the cancer cells. HER2 overexpression was indicated by either a 3+ (positive) or 2+ (equivocal) IHC score with fluorescence *in situ* hybridization (FISH) amplification (25). For patients with equivocal IHC scoring of HER2, a negative FISH result was a prerequisite. All patients of the VGH-TAYLOR study were enrolled between November 2018 and December 2021 from the Comprehensive Breast Health Center, Taipei Veterans General Hospital, a tertiary referral medical center at Taipei, Taiwan.

Samples and nucleic acid preparation. Formalin-fixed paraffin-embedded (FFPE) samples were collected after obtaining informed consent. For consistent results, a fixation time of 6-48 h in 10% neutral buffer formalin at room temperature for breast cancer biomarker testing was performed. At least seven unstained tumor sections with 10 μm in thickness were retrieved, with one used for hematoxylin and eosin (H&E) staining and six used for nucleic acid extraction. Hematoxylin (3.5 min at room temperature) was used for nuclear staining, and eosin (60 sec at room temperature) was used for cytoplasmic staining, allowing for differentiation of cellular components. H&E-stained slides were reviewed to ascertain the presence of adequate breast cancer cells (>70% of cancer composition).

Paraffin was removed by xylene and ethanol serial washes. Nucleic acid was extracted from 5-μm sections with the QIAmp DNA FFPE Tissue Kit (cat. no. 56404; Qiagen, Inc.) or AllPrep DNA/RNA FFPE Kit (cat. no. 80234; Qiagen, Inc.), while quality control and concentration were checked and determined using the Qubit fluorimeter (Invitrogen; Thermo Fisher Scientific, Inc.), Qubit dsDNA HS and Qubit dsDNA BR Assay Kits (cat. nos. Q32851 and Q32850; Thermo Fisher Scientific, Inc.). In the current study, treatment-naïve cancerous tissue was used for NGS. Targeted sequencing for the TruSight Oncology 500 (TSO500) panel was conducted on residual specimens from the VGH-TAYLOR study.

The storage conditions for the remaining nucleic acids after CGP aimed at maintaining their integrity for potential future use. Therefore, nuclear acid was stored at -80˚C in Tris-EDTA buffer or nuclease-free water. Repeated freeze-thaw cycles were avoided. A total of 120 pairs of DNA/RNA were tested for integrity and 108 passed quality control (QC) matrices for NGS experiments: QC parameters for TSO500 included DNA integrity number >7 and RNA integrity number >7, size distribution of 200-500 bp for library construction and >90% on-target enrichment with uniform coverage. Under optimal storage conditions with minimal nucleic acid degradation, a ~90% success rate (108 out of 120 paired DNA/RNA samples) in sequencing with quality metrics was reported.

Oncomine comprehensive panel. The Ion Torrent Oncomine Comprehensive Assay Panel v3 (OCP; cat. no. A35805; Thermo Fisher Scientific, Inc.) was used as the default CGP for the VGH-TAYLOR study, which enabled the detection of 161 cancer-related genes and the identification of single nucleotide variants (SNVs), CNVs, gene fusions and indels. A total of 10 ng of DNA and RNA sample input was required. Libraries were generated according to the standard protocols, which was constructed with the Ion AmpliSeq Library Kit Plus (cat. no. 4488990; Thermo Fisher Scientific Inc.), and were multiplexed for templating on the Ion OneTouch 2 System and subsequently sequenced on the Ion GeneStudio S5 Prime System (Thermo Fisher Scientific, Inc.) using the Ion 318 Chip Kit (cat. no. 488146; Thermo Fisher Scientific Inc.) with single-end semiconductor-based sequencing and a read length of 200 bp, according to the manufacturer's instructions (guide MAN0015885 Revision C of Thermo Fisher Scientific, Inc.). Sequencing data were analyzed, aligned and annotated through Torrent Suite v5.10.0 (Thermo Fisher Scientific, Inc.) and Ion Reporter v5.10 (Thermo Fisher Scientific, Inc.) software with the default Coverage Analysis (v5.10.0.3), Sample ID (v5.10.0.1) and Variant Caller (v5.10.0.18) plugin. Variants were further analyzed and interpreted for clinical actionability using the Oncomine Knowledgebase Reporter (Thermo Fisher Scientific, Inc.) database. The coverage metrics indicated that the number of mapped reads ranged from 4 to 6 million, with a mean depth of 1,100-1,600 times.

TSO500. TSO500 (cat. no. 20032626; Illumina, Inc.) was designed to identify known and emerging tumor biomarkers, using both DNA and RNA from tumor samples. These pan-cancer biomarkers aligned with key guidelines and clinical trials (22,23), including 523 genes for assessment of DNA and RNA variant types, plus microsatellite instability, tumor mutational burden and homologous recombination deficiency (optional). Libraries were prepared according to the manufacturer's guidelines from up to 80 ng DNA and 40 ng of RNA, using the TSO 500 library prep kit (cat. no. 20028216; Illumina, Inc.). Adapter ligation with unique molecular identifiers (UMIs) was performed with target fragments amplified and indexed. NGS was performed with a NextSeq 2000 sequencing system optimized for a minimum read length of 2x101 bp, operated by the Department of Pathology and Laboratory Medicine of the Taipei Veterans General Hospital using the NextSeq 1000/2000 P2 Reagents (300 cycles) v3 (Illumina, Inc.). Data were analyzed with the TSO500 Local App v2.2 (Illumina, Inc.), and variant call format files were further processed and annotated with the PierianDx software version CGW_v6.20 (PierianDx), which offered an integrated interpretation. The read collapsing analysis step executed an algorithm that collapses sets of reads (known as families) with similar genomic locations into representative sequences using UMI tags. Median exon fragment coverage across all exon bases was ≥150.

Benchmark comparisons. The difference in sequencing technology between amplicon-based (OCP) and hybrid capture-based (TSO500) methods has implications for detecting genetic variants, especially in homopolymer regions, consisting of a series of consecutive identical bases (26). Hybridization capture-based approaches show better uniformity, which can be relevant for detecting variants in homopolymer regions. Amplicon-based methods are faster and cost-effective but prone to errors in homopolymer regions. Hybrid capture methods are more reliable for these regions due to uniform coverage and reduced PCR bias, but they are more resource-intensive. Final reports from the medium- (OCP) and large-sized (TSO500) panels and accompanied tab-separated values files were collected. Variants reported from actionable genes were the primary endpoints in the current study. Clinical actionability was defined by the joint consensus of the Association for Molecular Pathology, American Society of Clinical Oncology and College of American Pathologists, published in 2017 (27). Additional annotations for actionability and OncoPrinter (28,29) visualization were conducted using the OncoKB database (30) and European Society for Medical Oncology (ESMO) Scale for Clinical Actionability of molecular Targets (ESCAT) criteria (11,12). Clinical actionability was categorized as follows: i) Tier I, actionability indicated an alteration-drug match associated with improved outcome

in clinical trials; ii) tier II, anti-tumor activity was associated with the matched alteration-drug but lacked prospective outcome data; and iii) tier III, the matched drug-alteration led to clinical benefit in another tumor type other than the tumor of interest. Polymorphisms were identified through population databases, such as the Single Nucleotide Polymorphism Database (31), the Genome Aggregation Database (32), 1000 Genomes Project and Exome Aggregation Consortium (33). Variants with minor allele frequency >1% were excluded. Variants outside the targeted regions of the sequencing panel were masked. Considering the sequencing depth, a 10% variant allele frequency (VAF) threshold assumed standard sequencing depths (500-1,000 times). The limit of detection was set to 5% for SNVs/indels and VAF <10% was considered a low-VAF status. An average copy number ≥4 was interpreted as a gain (amplification) and <1 as a loss (deletion). The concordance of filtered actionable genes (*AKT1*, *BRCA1/2*, *PALB2*, *ERBB2*, *PIK3CA* and *PTEN*) and *TP53* based on the original reports between the two panels was calculated (concordant variants divided by the sum of both concordant and discordant variants) and reported.

Data description and clinical usage. ESCAT-defined actionable genes, including fusions, amplifications, copy number gains, point mutations and indels, were extracted for downstream analysis. Categorical variables are presented as numbers and percentages. Official reports from the Ion Reporter and the Oncomine Knowledgebase Reporter, as well as those from Pierian Dx software were released to all participants when requested. In addition, primary care physicians received the same reports once they were available, augmenting clinical decision making.

Results

Study population and targeted actionable genes. A total of 108 patients with breast cancer from the VGH-TAYLOR study with adequate remaining nucleic acid (both DNA and RNA) were recalled. After explaining the purpose of this re-sequencing study, all participants signed informed consent forms and their specimens were assayed with the large-sized CGP, TSO500 panel. There were 54 patients in group 1A, 6 in group 1B, 25 in group 2, 5 in group 3-I, 7 in group 3-II and 11 biobank/retrospective cohort breast cancer samples. Early-stage breast cancer (groups IA and 2) constituted the majority of samples (73.1%, n=79). Four patients initially classified as TNBC at the time of enrollment were re-tested by FISH and found to be HER2-positive. Due to discrepancy in the interrogated genes (523 vs. 161 genes), only actionable genes listed by the ESCAT criteria for breast cancer were analyzed, as follows: i) Tier IA: *ERBB2* amplification, *BRCA1/2* germline mutation and *PIK3CA* mutation; ii) tier IC: *NTRK* translocation; iii) tier IIA: *PTEN* loss and *ESR1* mutation; iv) tier IIB: *AKT1* mutation and *ERBB2* mutation; v) tier IIIA: *BRCA1/2* somatic mutation and *MDM2* amplification; and vi) Tier IIIB: *ERBB3* mutation (11,12).

Mutational landscape of actionable genes with TSO500. Fig. 1A shows the mutational landscape of ESCAT-defined actionable genes among Taiwanese patients with TNBC examined with TSO500. Among 108 Taiwanese patients with breast cancer (all samples were TNBC except for four samples that were HER2-positive), *PIK3CA* was the most common actionable gene (39%, n=42), including fusion, amplification, copy number gain and point mutation, followed by *BRCA2*, including fusion, copy number gain, heterozygous loss, truncating and point mutation (24%, n=26). *BRCA1* variants, including fusions, truncating and point mutations were detected in 12% (n=13) of samples. *ERBB2* amplification, copy number gain, in-frame and point mutations were identified in 13% (n=14) of the study population. Importantly, among three breast cancer samples with HER2 amplification reported by TSO500, two coincided with the four clinically HER2-positive (overexpression) cases.

PTEN mutations including heterozygous/homozygous loss and truncating/in-frame/point mutations were reported in 15% (n=16) of breast cancer samples. Copy number gain and point mutations in *ERBB3* were identified in 10% (n=11) of the samples, while *ESR1* (2.8%, n=3; copy number gain/heterozygous loss and point mutation) and *MDM2* variants (1.9%, n=2; amplification and heterozygous loss) were infrequently mutated in the Taiwanese population. No *NTRK* translocations were reported. Table SI presents the actionable mutations assayed with TSO500 CGP.

Mutational landscape of actionable genes with OCP. Fig. 1B shows the mutational landscape of actionable genes reported by OCP. Compared with TSO500, OCP reported a higher number of *ERBB2* variants (19 vs. 13%), most of which came from higher proportion of missense mutations with unknown significance. Of the four clinical HER2-positive breast cancer samples, none reported a HER2 alteration, except for another HER2-equivocal case associated with an *ERBB2* I665V missense mutation, which was a variant of uncertain significance (VUS). Conversely, the frequencies of *BRCA1* and *BRCA2* variants were lower than those reported from TSO500 (*BRCA1*, 6 vs. 12%; and *BRCA2*, 5 vs. 24%). *PIK3CA* variants and *PTEN* mutations were also less frequent than those with TSO500 (28 vs. 39% and 6 vs. 15%, respectively). No alterations were identified by OCP in *ESR1*, *MDM2* and *ERBB3*. Table SII details the actionable mutations identified with OCP.

Comparisons of reported actionable variants between OCP and TSO500. For benchmark comparisons, the four HER2-positive samples were excluded, and the analysis was focused on tumor DNA sequence variants from actionable genes. Amino acid change (protein coordinate), genomic and transcript-dependent cDNA coordinates were used to identify variants reported as least once from either TSO500, OCP or both (Table I). Among 272 variants, 94 (34.6%) were identified by both platforms; therefore, the concordance rate was slightly higher than one-third based on the original reports. To understand the mechanisms underpinning the high discordance between TSO500 and OCP, a manual review of conflicting variants was conducted with all binary alignment map (BAM) files visualized through integrative genomic viewer by an author who is expert in precision oncology and bioinformatics (34). Fig. S1 provides such an example.

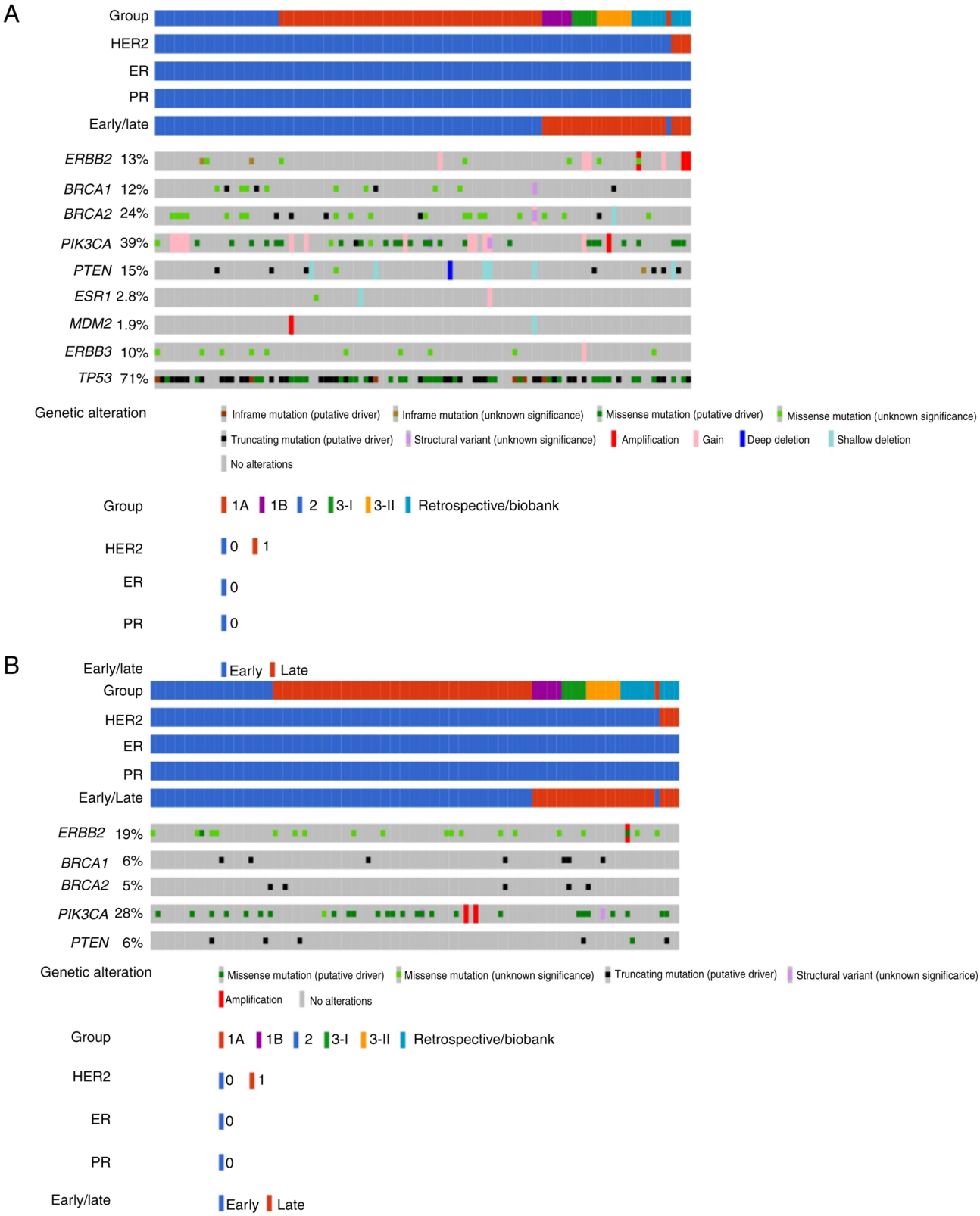

Figure 1. Mutational landscape of 108 Taiwanese patients with breast cancer assayed with (A) the TruSight Oncology 500 and (B) the Oncomine Comprehensive Assay Panel v3 for actionable genes. HER2, human epidermal growth factor receptor 2; ER, estrogen receptor; PR, progesterone receptor.

Fig. 2 shows the interpretation categories of the comparison: A total of 25 (9.2%) variants were the same with different annotations, 34 (12.5%) were beyond the OCP targeted regions, 21 (7.7%) were benign polymorphisms called by OCP but filtered out by TSO500, 15 (5.5%) were discordant variants with low variant allele frequency (VAF<10%) while TSO500 were more accurate in 83 cases (30.5%). After discarding out-of-targeted region variants, benign and low-VAF variants, the concordance rate approached 60% (119 of 202 variants, 58.9%) and the large-sized panel (TSO500) detected more

Table I. Variants of actionable genes and *TP53* (n=202) reported from both the TSO500 and OCP.

Gene	Variant	ESCAT	Both platforms	TSO500 only
AKT1	E17K	II-B	3	
BRCA1	S405*	III-A	1	
	R1203*	III-A	1	
	S1286fs	III-A	1	
	S632fs	III-A	1	
	c.5470-1G>A	III-A	1	
	G1350C	III-A		1
	M1783L	III-A		2
	N909S	III-A		1
	R1583K	III-A		1
	R762S	III-A		1
	S1389N	III-A		1
	V191I	III-A		1
	c.1A>G	III-A		1
BRCA2	S521*	III-A	1	
	E1571fs	III-A	1	
	N2135fs	III-A	1	
	C315S	III-A		1
	F2254Yfs*6	III-A		1
	G2508S	III-A		1
	G2901D	III-A		1
	H523R	III-A		1
	I1929V	III-A		4
	N72S	III-A		2
	P3292L, V2109I	III-A		1
	R2108C	III-A		5
	R2842H	III-A		1
	V2109I	III-A		1
	V2151Ffs*17	III-A		1
	V783A	III-A		1
ERBB2	L811V, L839R	II-B	1	
	L725S		1	
	P1140A			1
	V743_			1
	M744insHV			1
	Y742_ A745dup			1
NTRK1	K167R			1
	M530T			1
	R190Q			1
	R190W			1
NTRk3	D611E			2
PALB2	K353fs		1	
	A38G			1
	D498Y			2
	R825T			2

Table I. Continued.

Gene	Variant	ESCAT	Both platforms	TSO500 only
PIK3CA	E542K	I-A	3	
	E542K, E726K	I-A	1	
	E542Q, H1047R	I-A	1	
	E545K	I-A	3	
	E545Q, H1047Y	I-A	1	
	E726K		1	
	G1049R		1	
	H1047L	I-A	2	
	H1047R	I-A	9	
	N345I		1	
	Q546R		1	
	D350N			1[a]
	D725N			1[b]
PTEN	E150*	II-A	1	
	E43fs	II-A	1	
	P38fs	II-A	1	
	R130*	II-A	1	
	Q245*	II-A	1	
	c.1026+ 1G>A	II-A	1	
	V290Sfs*8	II-A		1
	G127_ G129del, G129E	II-A		1
TP53	C176R	IV-A	1	
	C242Afs	IV-A	1	
	C275Y	IV-A	1	
	E271*	IV-A	1	
	E56Kfs	IV-A	2	
	F109Sfs	IV-A	1	
	G108Vfs	IV-A	4	
	G245S	IV-A	2	
	H179R	IV-A	2	
	H179Y	IV-A	1	
	H193P	IV-A	1	
	H193R	IV-A	1	
	H193Y	IV-A	1	
	H214Qfs	IV-A	1	
	K132N	IV-A	1	
	L111Dfs, R196*	IV-A	1	
	L111Ffs	IV-A	1	
	L194H	IV-A	1	
	L252Hfs	IV-A	1	
	P151S	IV-A	1	
	Q192*	IV-A	1	
	R158Lfs	IV-A	1	
	R175H	IV-A	2	

Table I. Continued.

Gene	Variant	ESCAT	Both platforms	TSO500 only
	R196*	IV-A	2	
	R248Q	IV-A	4	
	R248W	IV-A	1	
	R273H	IV-A	4	
	R282W	IV-A	1	
	R333Vfs	IV-A	1	
	R342*	IV-A	1	
	S149Pfs	IV-A	1	
	S166*	IV-A	1	
	S241F	IV-A	1	
	T253Pfs	IV-A	1	
	V147*	IV-A	1	
	W146*	IV-A	1	
	W53*	IV-A	1	
	W91*	IV-A	1	
	Y103Afs	IV-A	1	
	Y107*	IV-A	1	
	Y205D	IV-A	1	
	c.560-2A>T	IV-A	1	
	c.993+1G>A	IV-A	1	
	c.994-2A>C	IV-A	1	
	c.993+1G>A, 526T>A	IV-A	1	
	c.920-2A>G	IV-A	1	
	c.993+1G>T	IV-A	1	
	c.560-1G>A	IV-A	1	
	c.919+1G>T	IV-A	1	
	c.993+2T>G	IV-A	1	
	c.993+1G>A	IV-A	1	
	D281_ K292del	IV-A		1
	E271Q	IV-A		1
	F113V	IV-A		1
	I251L	IV-A		1
	L265P	IV-A		1
	N131del	IV-A		1
	P190L	IV-A		1
	Q333E, R213*	IV-A		1
	R213*	IV-A		4
	T140_ C141 delinsS	IV-A		1
	V157_ R158insL	IV-A		1

[a]With additional H1047R noted by TSO500 and OCP; [b]with additional E453Q and E545K noted by TSO500 and OCP. OCP, Oncomine Comprehensive Assay Panel v3; TSO500, TruSight Oncology 500; ESCAT, European society for medical oncology Scale for Clinical Actionability of molecular Targets.

variants even for the same set of actionable genes and *TP53*. It deserves notice that certain variants were called by OCP but ignored by TSO500, which were proven to be homopolymer regions, including variants in *BRCA1* (n=2), *BRCA2* (n=2), *PALB2* (n=1) and *TP53* (n=3), as well as a misalignment with *PTEN* in one case and with *TP53* in two cases (data not shown).

Fig. 3A and B and Table II present concordant and discordant variants among actionable genes between two CGP panels. *AKT1* E17K exhibited a perfect concordance (100%, n=3), followed by *PIK3CA* (78%, n=29) and *TP53* (52%, n=49). With manual corrections of polymorphism and aliasing variants, the concordance of *TP53* (71%, n=67), *ERBB2* (69%, n=20), *PTEN* (55%, n=6), *BRCA1*, *BRCA2* and *PALB2* (29, 17 and 8%, respectively) was enhanced substantially. Most of the discordance came from those detected by TSO500 only, including variants in *BRCA1* (65%, n=11), *BRCA2* (83%, n=29) and *PALB2* (75%, n=9). Some variants were undetectable by OCP due to out-of-target regions or low VAF, which occurred in all *ERBB3* and *ESR1* variants, as well as *NTRK1* and *NTRK3* (76 and 71%, respectively) variants. True conflicting calling results were rare, including 3 *TP53* (3%), 2 *PIK3CA* (5%) and 1 *ERBB2* (3%) variants.

Discussion

To achieve the goal of personalized and precision medicine, CGP has been recognized as a key tool that can potentially transform cancer risk prediction, detection, diagnosis, treatment and monitoring. CGP can augment clinical decision-making in the form of either *in vitro* diagnostics (IVD) or companion diagnostics. Most importantly, the continuous price reduction across small- to large-sized CGP panels has improved patient access (35). Consequently, therapy assignments for newly diagnosed breast cancers and monitoring of patients who are in treatment are expected to benefit from CGP.

In the context of CGP, actionable genes refer to specific genetic alterations or mutations in a patient's (tumor) DNA that can guide therapeutic decisions or clinical interventions. These genetic changes are considered 'actionable' because they suggest potential treatments or clinical strategies that can directly impact the patient's care. This may include targeting specific mutations with precision medicine therapies, selecting clinical trials based on the genomic profile, such as basket or umbrella trials, or monitoring the patient for disease progression. Actionable genes typically include those that are associated with known therapies (such as targeted therapies and immunotherapies, including PARP inhibition for pathogenic *BRCA1/2* mutations), have established clinical guidelines for intervention based on their presence, or may offer prognostic or predictive value for disease outcomes, guiding treatment options (1-5). In the present study, the performance of medium- to large-sized gene panels in a TNBC patient cohort was compared. Both OCP and TSO500 are used worldwide, but there are few direct comparison studies for breast cancer actionable genes in the literature (18,19,36,37).

The four HER2-positive breast cancer cases provided an opportunity to evaluate the association between clinical HER2-status and NGS-based *ERBB2* CNVs. None of these cases were reported as HER2-amplified by OCP while two of three HER2-amplified cases reported by TSO500 coincided

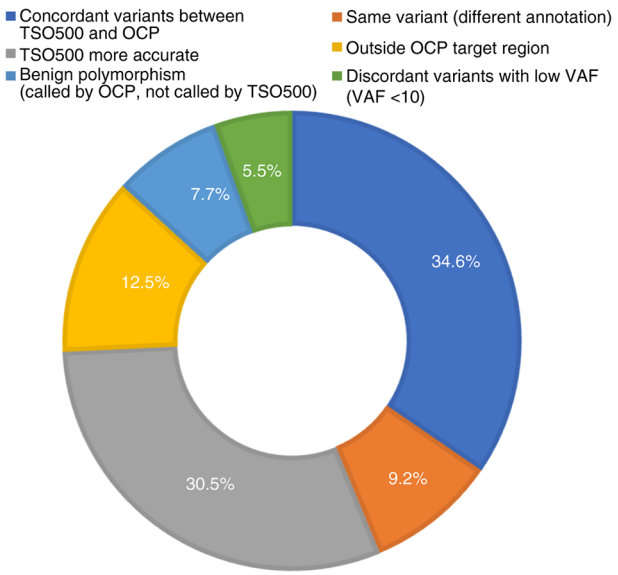

Figure 2. Interpretation categories of 272 variants called at least once by either TSO500, OCP or both. TSO500, TruSight Oncology 500; OCP, Oncomine Comprehensive Assay Panel v3; VAF, variant allele frequency.

with the four clinically HER2-positive breast cancers. A higher number of HER2-gained breast cancers were identified by TSO500, indicating the potential for anti-HER2 targeting therapy. As *NTRK* translocation was not listed as the targeted alteration of OCP, the detectability of structure variants (CNV and fusion) was not compared further.

In the present study, the genes and variants reported from either OCP v3 or TSO500 when genomic, cDNA and protein coordinates were used for variant sorting were detailed. This subset represented the most actionable variants revealed by medium- and large-sized CGP. *AKT1* is an intra-cellular kinase and is predominantly altered in breast and endometrial cancer. The *AKT1* E17K is the most common alteration across various tumor types, including breast and gynecological cancers (38,39). The CAPItello-291 phase III trial demonstrated that capivasertib (AZD5363) and fulvestrant combination therapy resulted in markedly longer progression-free survival compared with the placebo and fulvestrant groups (40). The three *AKT1* E17K-mutant cases in the present study were identified by both panels.

There were two *BRCA1* (S405* and R1203*) and one *BRCA2* (S521*) truncating mutations, and reflex germline testing should precede if PARP inhibitor was considered for these patients, as only germline *BRCA1/2* mutations are actionable in breast cancer (41). The *BRCA1* c.5470-1G>A SNP has been recognized as pathogenic by both the Breast Cancer Information Core and Consortium of Investigators of Modifiers of BRCA1/2 (42,43). The *BRCA2* S521* truncating mutation impairs nuclear localization of BRCA2, which is essential for normal BRCA2 function (44). Despite common truncating and frameshift mutations (five for *BRCA1* and three for *BRCA2*) called by both panels, only TSO500 revealed more suspicious variants (eight variants among 9 cases for *BRCA1* and 13 variants among 21 subjects), while OCP was flawed by spurious mutations (*BRCA1*: S1180fs and T1376fs; and *BRCA2*: S538fs, T598fs, S973fs, E33*, T912fs, S3041fs and S3147fs).

PALB2 K353fs is a truncating mutation in a tumor suppressor gene and therefore is likely oncogenic. The phase II TBCRC 048 study also indicated the predictive power of the germline *PALB2* mutation for metastatic breast cancer, as a high response rate (82%) was observed when olaparib, a PARP inhibitor, was used (45). In the present study, TSO500 identified three additional variants in 5 cases while OCP reported 1 false positive case with up to four variants (D1125fs, N368fs, S357fs and N342fs). Tumor-only sequencing made differentiating germline from somatic *BRCA1/2* and *PALB2* mutations challenging. However, LOH-germline inference calculator and the somatic-germline-zygosity algorithms helped distinguish these mutations, with one somatic and eight germline *BRCA1/2* mutations identified (46).

One patient harbored two mutations in *ERBB2* (L811V and L839R) and another with the L725S mutation was recognized by both platforms, none of which were among the oncogenic mutations approved by U.S. Food and Drug Administration for the use of neratinib, a pan-HER kinase inhibitor that binds irreversibly to the ATP-kinase domain of HER2 inhibiting the downstream phosphorylation of AKT and MAPK (47). TSO500 identified three additional *ERBB2* mutations (P1140A, V743_M744insHV and Y742_A745dup). It is important to note that TSO500 detected more complex mutations (such as Y742_A745dup and V743_M744insHV, both classified as VUS) than did OCP.

Certain *PIK3CA* hotspot mutations have been identified from the SOLAR-1 trial for the usage of alpelisib, a selective PI3K-α inhibitor, namely C420R, E542K, E545A, E545D, E545G, E545K, Q546E, Q546R, H1047L, H1047R and H1047Y (48). Our previous study also observed double mutations of *PIK3CA* among the Taiwanese population (21). Only four cases with E726K, G1049R, N345I and Q546R mutations were outside the hotspot region, while two cases with D350N or D725N were co-mutant with other hotspots, which were only reported by TSO500. Consequently, both platforms identified the same number of *PIK3CA*-altered breast cancer cases.

Conversely, selective PI3K-β inhibitors, GSK2636771 and AZD8186, are ATP competitors and have shown preclinical antitumor response and durability for *PTEN*-deficient solid tumors including TNBC (49). The *PTEN* c.1026+1G>A SNP has been reported as clinically pathogenic at least twice (Invitae Clinical Genomics Group and ClinGen PTEN Variant Curation Expert Panel), with germline origin (50,51). The remaining five consensual variants (E150*, E43fs, P38fs, R130* and Q245*) were truncating mutations; TSO500 identified two additional cases with *PTEN* mutations (G127_G129del and V290Sfs*8) and OCP called one inappropriate variant due to misalignment (c.386G>A).

Quite a few *TP53* alterations were identified by both OCP and TSO500. Despite not being currently druggable, *TP53* may act as an independent prognostic factor for early and advanced-stage cancer with a wide range of mutational frequency (52). It has been argued that all *TP53* mutations from tumor-only sequencing are somatic, and rarely representative of the germline Li-Fraumeni syndrome (53). TSO500 identified more variants than OCP did, while the latter reported three cases with V73fs in homopolymer region and one case with misaligned V73fs mutation, both of which were spurious.

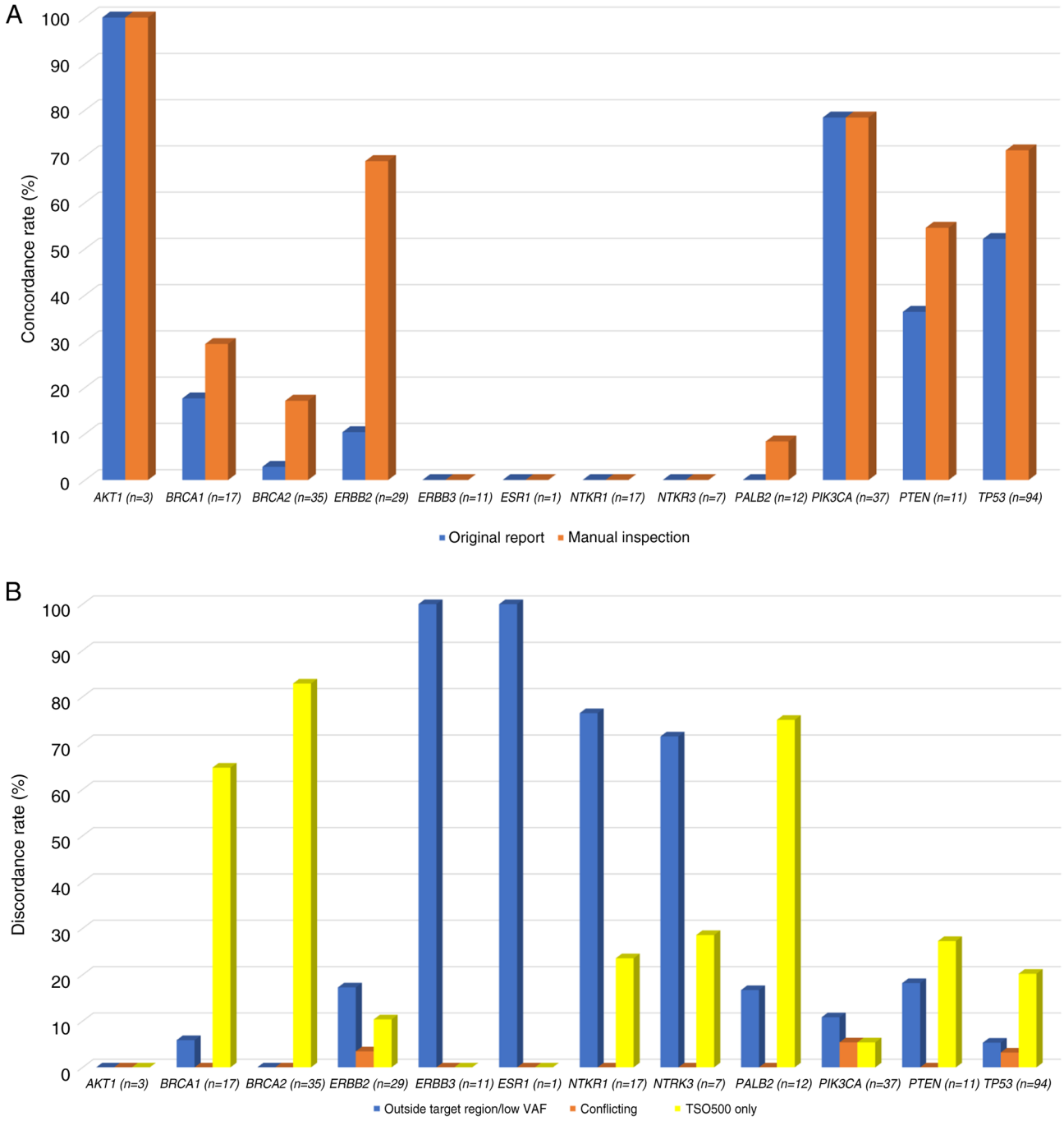

Figure 3. (A) Concordance and (B) discordance distributions among variants of actionable genes and *TP53* between the TSO500 and the Oncomine Comprehensive Assay Panel v3. TSO500, TruSight Oncology 500; VAF, variant allele frequency.

A total of 272 variants were identified among actionable genes and *TP53* from at least one panel. Interestingly, the concordance rate from original reports was low at slightly more than one-third (34.6%). With extensive bioinformatics analyses and manual curation, three-fifths of concordance (58.9%) were achieved after exclusion of unfiltered polymorphisms, low-VAF variants and those beyond the targeted scope of OCP. Among inconsistent variants, some were identical after manual inspections, further indicating the necessity of analytical abilities and domain knowledge in genomic nomenclature (54). Furthermore, an additional 62 variants were revealed by TSO500, indicating missed opportunities by OCP, which may be explained by fundamental discrepancies in sequencing technology. The accuracy of the amplicon-based OCP vs. the hybrid capture-based TSO500 panel can be influenced by their underlying methodologies. Amplicon-based methods utilize PCR amplification with sequence-specific primers to enrich target regions. By contrast, hybrid capture sequencing employs hybridization-based capture to enrich for desired genomic regions. Hybrid capture may offer a more robust and accurate approach, especially for clinical applications (55).

In the context of TNBC research, a VAF cut-off of <10% is used to filter out low-confidence or likely spurious SNVs identified through NGS of tumor samples (56,57). The

Table II. Deconvolution of variants among actionable genes and *TP53* with concordant (original report and manual inspection) and discordant (outside targeted region/low VAF), conflicting results and TSO500-detected only results.

	AKT1 (n=3)	*BRCA1* (n=17)	*BRCA2* (n=35)	*ERBB2* (n=29)	*ERBB3* (n=11)	*ESR1* (n=1)	*NTKR1* (n=17)	*NTRK3* (n=7)	*PALB2* (n=12)	*PIK3CA* (n=37)	*PTEN* (n=11)	*TP53* (n=94)
Original report	100	18	3	10	0	0	0	0	0	78	36	52
Enhanced with manual inspection	100	29	17	69	0	0	0	0	8	78	55	71
Outside targeted region/low VAF	0	6	0	17	100	100	76	71	17	11	18	5
Conflicting result	0	0	0	3	0	0	0	0	0	5	0	3
TSO500-detected only	0	65	83	10	0	0	24	29	75	5	27	20

The values are presented as percentages. TSO500, TruSight Oncology 500; VAF, variant allele frequency.

threshold may be beneficial to eliminate technical, background noise and subclonal mutations, as tumor samples can be contaminated with normal cells, and low-level DNA damage or degradation can introduce background noise that interferes with accurate SNV calling. In addition, tumors are often composed of multiple subclones with distinct genetic profiles. SNVs present in minor subclones may have VAFs <10%, making them difficult to distinguish from background noise (58). A VAF cut-off of 5-10% is commonly used in various cancer genomics studies, including those focused on TNBC. This range is considered to achieve a reasonable balance between sensitivity (detecting true SNVs) and specificity (excluding false positives) (59).

Despite being one of the pioneers to directly compare two commercialized targeted panels, there were some limitations to the present study. First, sequencing was not conducted concurrently, and the 1-2 year delay of the TSO500 analysis following OCP may have introduced some bias from nucleic acid degradation, despite all samples being stored under temperature-controlled conditions. Second, variants called by each panel were considered based on the standard or formal algorithm of each platform (Ion Reporter/Oncomine Knowledgebase Reporter for OCP and PierianDx for TSO500), which limited the comparability between distinct platforms. For unbiased comparison, the same aligner, caller and annotator should be applied. However, browser extensible data files were unavailable from manufacturers to confirm the jointly interrogated regions. On the other hand, all commercial CGP solutions are under regulation as either laboratory developed tests or IVD, and practically these pre-set algorithms should not be modified arbitrarily to enhance reproducibility. Despite this, the present study conducted an exhaustive bioinformatic analysis from BAM files to dissect the conflicting results from the same samples. Third, *NTRK* is not within the targeted fusion genes of OCP; therefore, a comparison with TSO500 was not possible. Moreover, *NTRK* sequence variants, amplifications or fusions are not actionable for breast cancer. As larotrectinib and entrectinib are approved in a number of countries, *NTRK* fusion as a tumor-agnostic marker is important, and its detection should be incorporated into CGP for breast cancer (60). Fourth, as most cases are triple negative phenotypically, a cohort of ~100 patients with TNBC is suitable for a comparative sequencing panel study. However, if the goal is to perform more detailed subgroup analyses or identify rare mutations, a larger cohort may be more appropriate. In the future, conducting a prospective and parallel study across distinct CGP platforms could reveal whether larger sequencing panels justify their higher costs by improving the detection of actionable mutations, refining novel therapeutic strategies, and characterizing the complex genomic landscape of TNBC. Furthermore, it would provide critical insights to optimize genomic profiling for other breast cancer subtypes, advancing both research and clinical care.

As an observational study, the present study could not directly assess the impact of CGP on treatment outcomes, as no intervention was performed. During the study period, the compassionate use of alpelisib (a selective PI3K inhibitor) benefited nine patients, and the out-of-pocket use of olaparib (a PARP inhibitor) benefited two patients, demonstrating the actionability of the testing. Since May 2024, NGS, specifically

whole-exome sequencing for *BRCA1/2*, has been reimbursed in Taiwan for patients with stage II or higher TNBC. The Regulations of Special Medical Techniques of Taiwan require that all NGS testing results (including both whole-exome sequencing and targeted panels) be submitted to the National Health Research Institutes Biobank. It is anticipated that cost-effective implementation of larger NGS panels in routine clinical practice will be possible in the future, once sufficient data from NGS results and accompanying clinical outcomes are available.

In conclusion, the present study comparing medium- and large-sized sequencing panels in a cohort of patients with TNBC may provide critical insights into the balance between actionable gene findings, cost and complexity. TSO500, the larger panel, detected more variants than OCP did, even from the same set of ESCAT-defined actionable genes and *TP53*. Conversely, a proportion of inconsistent variants could be manually curated and were identical with aliasing coordinates or starting positions. Finally, there were variants detectable only by TSO500, indicating potential and fundamental differences in sequencing technology, bioinformatic algorithms and variant filtering. The value of a large-sized panel in clinical usage is ascertained, considering the beneficiaries of PARP inhibition for the higher number of *BRCA1/2* and *PALB2* mutations detected, as indicated by the National Comprehensive Cancer Network and ESMO guidelines (61,62). Given the experience from the present study, the updated Oncomine Comprehensive Assay Plus with >500 genes profiled is anticipated in future studies (63). The results could guide panel selection for precision oncology, ensuring optimal clinical outcomes while maximizing resource efficiency. This approach would be particularly impactful in refining strategies for TNBC management.

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Availability of data and materials

The data generated in the present study may be found in the Sequence Read Archive database under accession number PRJNA1256731 or at the following URL: https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1256731.

Authors' contributions

CCH and LMT conceived the study. CCH drafted the manuscript. YCY conducted bioinformatics analyses. YFT, YSL, TCC and CYL collected samples and certified the NGS reports. HLH conducted NGS experiments. CCH and YCY confirm the authenticity of all the raw data. LMT approved the final submission. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was reviewed and approved by the Institutional Review Board of Taipei Veterans General Hospital (approval nos. 2021-01-007B and 2023-08-002B; Taipei, Taiwan). Participants provided written informed consent for both the prospective/retrospective VGH-TAYLOR study (approval no. 2021-01-007B) and for the retrospective re-sequencing study (approval no. 2023-08-002B).

Patient consent for publication

All participants agreed to the publication of the present study, with all identifying information removed.

Competing interests

The authors declare that they have no competing interests.

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