RESEARCH ARTICLE

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Performance evaluation of an amplicon-based next-generation sequencing panel for *BRCA1* and *BRCA2* variant detection

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Abstract

Background: As next-generation sequencing (NGS) technology matures, various amplicon-based NGS tests for *BRCA1/2* genotyping have been introduced. This study was designed to evaluate an NGS test using a newly released amplicon-based panel, AmpliSeq for Illumina BRCA Panel (AmpliSeq panel), for detection of clinically significant *BRCA* variants, and to compare it to another amplicon-based NGS test confirmed by Sanger sequencing.

Methods: We reviewed *BRCA* test results done by NGS using the TruSeq Custom Amplicon kit from patients suspected of hereditary breast/ovarian cancer syndrome (HBOC) in 2018. Of those, 96 residual samples with 100 clinically significant variants were included in this study using predefined criteria: 100 variants were distributed throughout the *BRCA1* and *BRCA2* genes. All target variants were confirmed by Sanger sequencing. Duplicate NGS testing of these samples was performed using the AmpliSeq panel, and the concordance of results from the two amplicon-based NGS tests was assessed.

Results: Ninety-nine of 100 variants were detected in duplicate *BRCA1/2* genotyping using the AmpliSeq panel (sensitivity, 99%; specificity, 100%). In the discordant case, one variant (*BRCA1* c.3627dupA) was found only in repeat 1, but not in repeat 2. Automated nomenclature of all variants, except for two indel variants, was in consensus with Human Genome Variation Society nomenclature.

Conclusion: Our findings confirm that the analytic performance of the AmpliSeq panel is satisfactory, with high sensitivity and specificity.

KEYWORDS
amplicon-based panel, *BRCA1*, *BRCA2*, evaluation, next-generation sequencing

1 | INTRODUCTION

In 1994, linkage analysis in large numbers of families identified *BRCA1* and *BRCA2* as genes associated with predisposition for hereditary breast/ovarian cancer syndrome (HBOC).[1,2] Approximately 5%-10% and 20% of breast and ovarian cancer cases are considered hereditary tumors,[3,4] but only 25% of HBOC are associated with *BRCA1/2* pathogenic variants, which affect DNA repair mechanisms.[5] Carriers with *BRCA1/2* pathogenic variants have a higher risk of developing breast cancer (60%-85%) and ovarian cancer (15%-40%) over their lifetime. [6,7] In *BRCA*-mutated patients, both intensive screening (including MRI) and prophylactic surgery or chemical treatment decrease cancer risk and overall mortality.[8-10] Among triple-negative breast cancer patients, platinum-based chemotherapeutic agents are favorable for *BRCA1/2* variant carriers.[11] Recently, poly ADP-ribose polymerase (PARP) inhibitors were reported to improve prognosis in patients with *BRCA*-mutated metastatic ovarian cancer.[12] Collectively, these reports indicate that testing for *BRCA1/2* mutation plays a significant role in the choice of therapy, as well detection of the genetic cause.

Next-generation sequencing (NGS) was introduced to clinical laboratories for multi-gene and high-throughput analysis.[13] Subsequently, NGS has been developed as a powerful tool for detecting *BRCA1/2* variants.[14-17] Although the high performance and cost-effectiveness of the NGS technique are well known, the diversity of NGS platforms, enrichment methods, and analytic pipelines represents a potential obstacle to implementation. Because amplicon-based methods for enrichment have several strengths, including lower cost, shorter preparation time, and smaller quantities of input DNA in comparison with capture methods,[18] several *BRCA1/2* NGS tests using amplicon methods have been developed.[19-23] NGS-based *BRCA1/2* variant tests have mainly been validated by Sanger sequencing, which is still considered to be the gold standard. This study was designed to evaluate the AmpliSeq for Illumina BRCA panel (AmpliSeq panel), an amplicon enrichment method for NGS testing, for detection of clinically significant BRCA variants, confirmed by Sanger sequencing, that were detected by another amplicon enrichment method, the TruSeq Custom Amplicon kit (TruSeq kit).

2 | MATERIALS AND METHODS

The Institutional Review Board/Ethics Committee of Asan Medical Center waived the requirement for informed consent for this study (2019-0044).

2.1 | Sample selection and DNA extraction

This study was performed at a single center. In 2018, 883 patients diagnosed with breast or ovarian cancer suspected to be HBOC were tested clinically for *BRCA1/2* variants by NGS using the TruSeq Custom Amplicon kit (Illumina) and Illumina MiSeqDx (Illumina) at our center. Genomic DNA was extracted from peripheral blood using the QIAGEN QIAamp DNA Mini Kit (QIAGEN). Of the reported variants from these 883 patients, 100 target variants were included in this study, based on the following criteria: (a) variants should be dispersed throughout the *BRCA1/2* genes, and (b) variants should be clinically significant (pathogenic, likely pathogenic, or variant of uncertain significance [VUS] based on the interpretation guideline from the American College of Medical Genetics and Genomics and the Association for Molecular Pathology).[24] All target variants were confirmed by Sanger sequencing. Ninety-six genomic DNA samples comprising 100 target variants were collected with anonymization. A schematic workflow of this study is shown in Figure 1.

2.2 | AmpliSeq panel-based NGS

A single NGS platform, MiSeqDx, was adopted for the detection of small indel and single-nucleotide variants. The AmpliSeq for Illumina
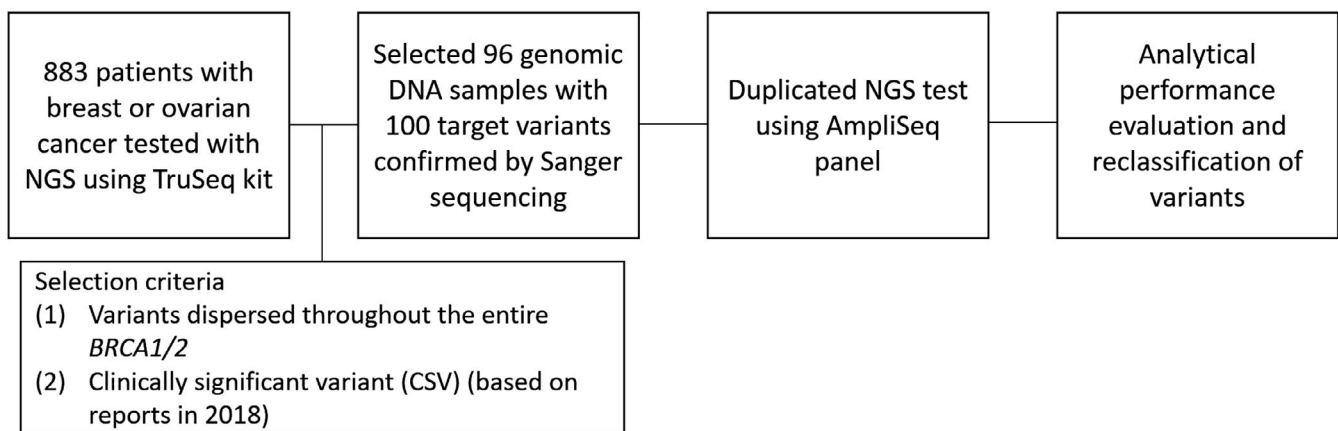

FIGURE 1 Schematic flowchart of the study design. Clinically significant variants included pathogenic variants, likely pathogenic variants, and variants of unknown significance. Abbreviation: TruSeq kit, TruSeq Custom Amplicon kit; AmpliSeq panel, AmpliSeq for Illumina BRCA panel

BRCA panel (Illumina), which contains 265 amplicons with average amplicon length of 98 bp, covers 22 404 base pairs, including all exons of *BRCA1/2*. Experiments using the AmpliSeq panel were performed in four separate batches containing 24 samples and repeated to confirm reproducibility.

2.3 | Bioinformatic analysis

Human genome build 19 (hg19) was used for alignment. Analysis was performed with the Illumina MiSeq Reporter using the following software: DNA Amplicon BaseSpace Workflow 2.00, DNA Amplicon Workflow 3.23.7.3 + master, BWA-MEM Whole-Genome (aligner) 0.7.12-r1039, Pisces Variant Caller 5.2.9.22, Illumina Annotation Engine 2.0.11-0-g7fb24a09, Bam Metrics v0.0.22, and SAMtools 1.2. Variants were filtered and annotated with Variant Studio. All variants were described according to the recommendation of the Human Genome Variation Society (https://www.hgvs.org/) using the reference transcript sequences of NM_007294.3 and NM_000059.3 for *BRCA1* and *BRCA2*, respectively. The target variants were confirmed using Integrative Genomic Viewer (IGV) (http://software.broadinstitute.org/software/igv). Along with the evaluation of this panel, the detected variants were reclassified based on the interpretation guidelines from the American College of Medical Genetics and Genomics and the Association for Molecular Pathology.[24]

2.4 | Statistical analysis

To evaluate performance, the results from NGS using AmpliSeq panel were compared with the NGS results obtained using TruSeq kit and confirmed by Sanger sequencing. Sensitivity, specificity, negative predictive value, and positive predictive value were determined, and 95% confidence intervals were calculated using the efficient-score method. The NGS test using AmpliSeq panel was performed in duplicate to examine reproducibility.

3 | RESULTS

3.1 | Target variants

We analyzed a total of 100 variants, comprising 66 single-nucleotide variants and 34 indel variants. Characteristics of these variants are shown in Figure 2, and all variants are listed in Table S1. Only two
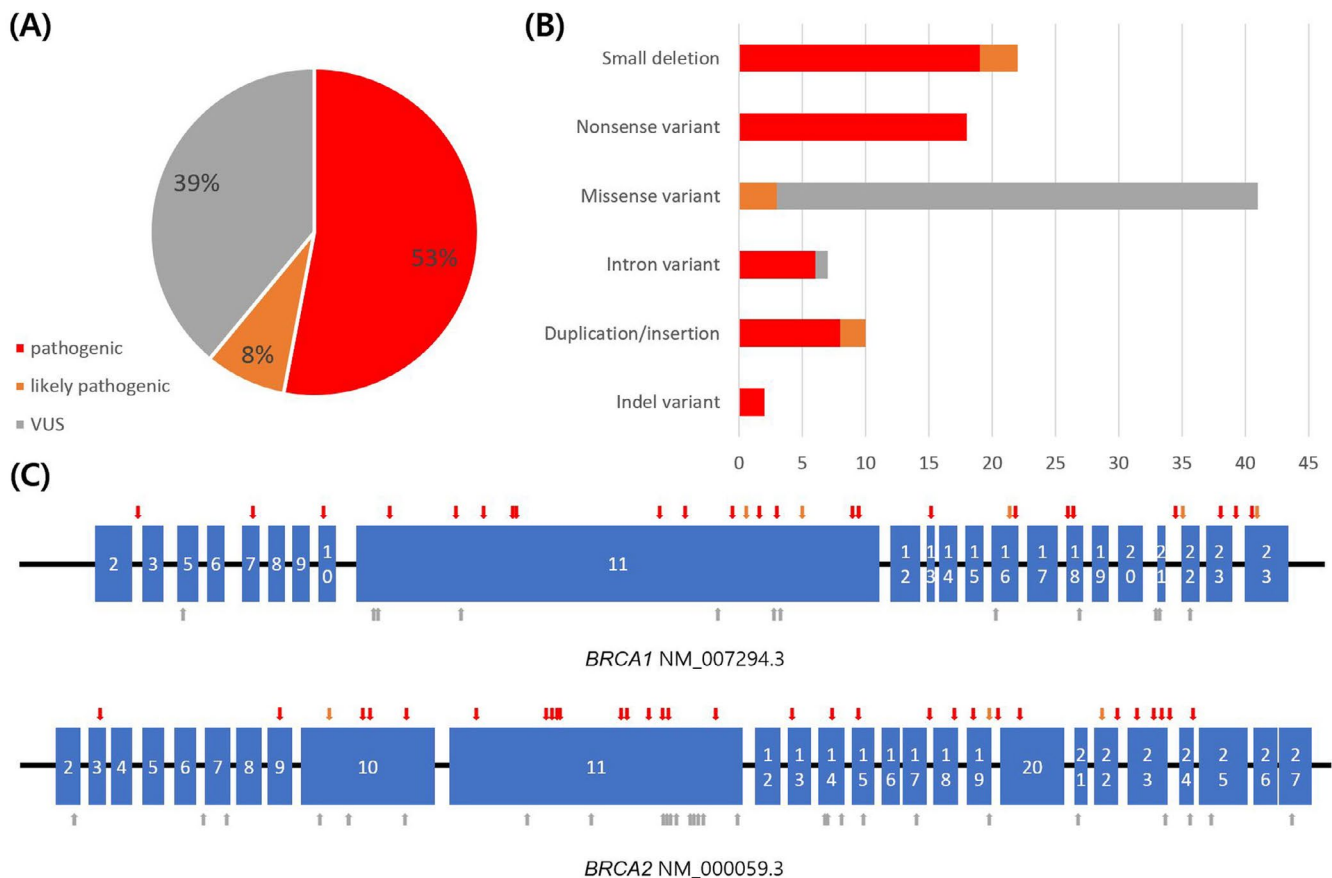

FIGURE 2 Types and distribution of clinically significant variants (n = 100) on *BRCA1* and *BRCA2* exons. A, Variant classifications, B, Variant types, C, Variant locations. Upper arrows indicate pathogenic or likely pathogenic variants, and lower arrows indicate VUS. Exon 4 was omitted because of a revision made after the initial description. Abbreviation: VUS, variants of unknown significance

TABLE 1 Run statistics of sequencing using AmpliSeq for Illumina BRCA

	Batch 1		Batch 2		Batch 3		Batch 4	
	Repeat 1	Repeat 2	Repeat 1	Repeat 2	Repeat 1	Repeat 2	Repeat 1	Repeat 2
On-target reads, %	96.65	96.57	96.65	96.53	96.68	96.55	96.63	96.48
Percent Q30 bases	96.41	94.73	94.57	96.10	95.81	94.39	95.82	95.86
Coverage at 20X, %	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Uniformity of base coverage at 0.2, %	99.98	100.00	99.95	100.00	100.00	100.00	100.00	100.00
Average depth per sample (min, max)	1410.5 (1085.9, 1838.6)	1432.2 (776.5, 3466.3)	1462.1 (1075.6, 1950.6)	1476.4 (928.6, 1915.8)	1481.0 (996.8, 2101.7)	1778.1 (1367.1, 3008.8)	1521.6 (963.6, 2386.0)	1569.1 (1059.4, 1931.6)

variants (*BRCA1* c.5496_5506delinsA, *BRCA2* c.9309A > G) were found in two different samples, and all of the other variants were found in one sample.

3.2 | Technical performance

A total of eight runs were performed: four batches, each containing 24 samples, were repeated. The quality control (QC) parameters of sequencing using the AmpliSeq panel were acceptable for all runs. The specific values of QC parameters are listed in Table 1.

3.3 | Analytical performance

All target variants except one were successfully detected by the AmpliSeq panel. Sensitivity, specificity, positive predictive value, and negative predictive value were 99%, 100%, 100%, and ~100% (95% confidence interval: 93.8%-99.90%, 100.0%-100.0%, 95.3%-100%, and 100%-100%), respectively, between the two NGS kits (Table 2). In *BRCA1*, one small duplication variant was not called from repeat 2 of one sample in batch 4. No discordant variants were found in *BRCA2*. Reproducibility of the AmpliSeq panel was 99.0% (100.0% for batches 1%-3% and 95.8% for batch 4).

In the discordant case, one variant (*BRCA1* c.3627dupA) was called only in repeat 1, but not in repeat 2. However, the variant was

visible with low variant allele frequency (VAF) (19.4%) (Figure 3). After detailed review, we determined that all sixteen variants except one (from another sample in the same batch) were called: The exceptional case was detectable only on IGV due to low VAF. These observations suggested a possible error in sample preparation.

3.4 | Variant annotation

After reclassification, eight variants from 39 target variants previously classified as VUS were designated as benign or likely benign. All were missense variants: five in *BRCA1* and four in *BRCA2*. Reclassification was mainly due to observations with a pathogenic variant. Thus, 31 variants remained as VUS. These are also listed in Table S1.

The nomenclature of all but two of the detected target variants was consistent with HGVS recommendations. In the two exceptional cases, indel variants (*BRCA1* c.922_924delAGCinsT and *BRCA1* c.5496_5506delinsA) were detected as two individual variants (one insertion variant and one deletion variant). These variants were observed in *cis* after sequence confirmation on IGV and were therefore reclassified as single indel variants.

4 | DISCUSSION

The genetic diagnosis of breast and ovarian cancer is crucial for genetic counseling, surveillance, and tailored treatment. NGS-based

TABLE 2 Concordance of target variants between AmpliSeq for illumina BRCA and the TruSeq Custom Amplicon kit confirmed by Sanger sequencing

AmpliSeq panel	TruSeq Custom Amplicon kit confirmed by Sanger sequencing		Notes
	Detected	Not detected	
Detected	99 true positives	0 false positive	99% sensitivity (95% CI: 93.8%-99.90%)
Not detected	1 false negative	22.4 Mbp true negatives	100% specificity (95% CI: 100%-100%)

Note: In one false-negative case, the variant was found in repeat 1 of AmpliSeq panel testing, but not called in repeat 2 due to low variant allele frequency.
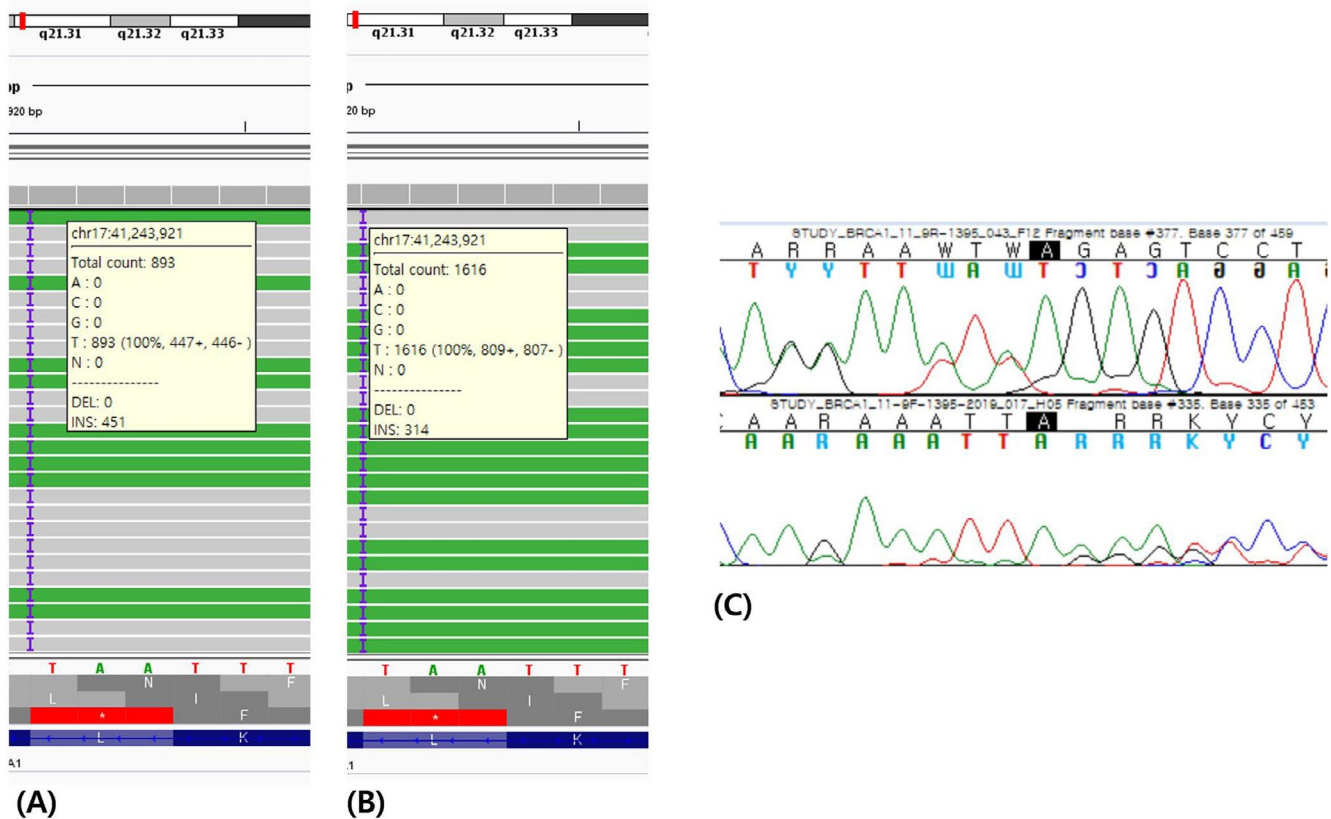
FIGURE 3 Integrative genomic viewer and chromatogram of a duplication variant from a discordant case. A, First repeat of AmpliSeq panel-based NGS. B, Second repeat of AmpliSeq panel-based NGS. C, Chromatogram of Sanger sequencing

variant testing has emerged as a powerful tool for *BRCA1/2* gene testing. Therefore, several studies have validated NGS techniques for *BRCA1/2* testing. This study is the first to validate the performance of the AmpliSeq for Illumina BRCA panel for clinical application. Based on a comparison with the TruSeq kit confirmed by Sanger sequencing, our findings suggest that the analytical performance of the AmpliSeq panel is acceptable for detection of *BRCA1/2* variants.

In this study, the target variants were well dispersed throughout *BRCA1/2*. Germline variants of *BRCA1/2* are well known for their wide distribution.[25,26] Subsequently, widely distributed variations with visual confirmation are needed to adequately evaluate the performance of *BRCA1/2* variant testing. However, a functional study reported that more variants occur in the RING domain, exon 11-13, and the BRAT domain of *BRCA1*[27]; consistent with that, almost all *BRCA1* target variants (97.5%) were in these regions in this study.

The sensitivity and specificity of the AmpliSeq panel were 99% and 100%, respectively, and the one discordant case was probably due to a mistake in sample preparation. Therefore, this panel was nearly equivalent to the previously adopted NGS kit used for comparison, as well as Sanger sequencing. Moreover, the high reproducibility of the panel demonstrated its reliability. Other validation studies regarding NGS-based *BRCA* testing revealed analytical specificity of 95.9%-100% and analytical sensitivity of 100%.[19,20,28] Collectively, these findings confirm the high performance of NGS-based *BRCA1/2* testing.

In regard to the error in sample preparation, we note that the QC results from this run were acceptable. To avoid such errors, detected variants should be compared with variants found in other samples from the same run, and abnormal samples should be re-examined. Other validation studies reported limitations due to technical factors such as low average coverage depth.[19,28] In addition to those sources of errors, procedural errors, such as in our case, do occur (albeit rarely) in the clinical laboratory. Therefore, this report emphasizes the need for clinical laboratories to make their best efforts to decrease errors in procedures.

In this study, indel variants were separated into insertion and deletion variants, mandating visual confirmation of whether the two variants were in *cis* or *trans*. Variant calling errors frequently arise for indel variants. Accordingly, we need to confirm all variants manually for accurate reporting of indel variants.

Our results indicated that the panel performed well but was limited by the low abundance of copy number variations (CNVs). In the Korean population, CNVs are less frequent than in other populations[29,30]; the CNV frequency in Korean familial breast cancer patients ranges from 0.44% to 0.8%.[31,32] However, a novel NGS test for detection of CNVs in *BRCA1/2* is needed. Second, because this study was not a diagnostic cohort study, its clinical validity could not be investigated. However, we could adequately evaluate the analytic performance of this panel because we chose target variants widely dispersed throughout *BRCA1/2*.

In conclusion, this study shows that the analytic performance of AmpliSeq panel is satisfactory, with high sensitivity and specificity. Therefore, the AmpliSeq panel performs sufficiently well to be implemented in the clinical laboratory for detection of *BRCA1/2* variants. Further improvement in testing and bioinformatic platforms will be required to overcome the remaining limitations with regard to detection of CNVs detection and calling and annotation of indel variants.

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REFERENCES

1. Miki Y, Swensen J, Shattuck-Eidens D, et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science*. 1994;266(5182):66-71.
2. Wooster R, Neuhausen SL, Mangion J, et al. Localization of a breast cancer susceptibility gene, BRCA2, to chromosome 13q12-13. *Science*. 1994;265(5181):2088-2090.
3. Campeau PM, Foulkes WD, Tischkowitz MD. Hereditary breast cancer: new genetic developments, new therapeutic avenues. *Hum Genet*. 2008;124(1):31-42.
4. Walsh T, Casadei S, Lee MK, et al. Mutations in 12 genes for inherited ovarian, fallopian tube, and peritoneal carcinoma identified by massively parallel sequencing. *Proc Natl Acad Sci*. 2011;108(44):18032-18037.
5. Nielsen FC, van Overeem HT, Sorensen CS. Hereditary breast and ovarian cancer: new genes in confined pathways. *Nat Rev Cancer*. 2016;16(9):599-612.
6. Thompson D, Easton DF. Cancer Incidence in BRCA1 mutation carriers. *J Natl Cancer Inst*. 2002;94(18):1358-1365.
7. Brose MS, Rebbeck TR, Calzone KA, Stopfer JE, Nathanson KL, Weber BL. Cancer risk estimates for BRCA1 mutation carriers identified in a risk evaluation program. *J Natl Cancer Inst*. 2002;94(18):1365-1372.
8. Hartmann LC, Sellers TA, Schaid DJ, et al. Efficacy of bilateral prophylactic mastectomy in BRCA1 and BRCA2 gene mutation carriers. *J Natl Cancer Inst*. 2001;93(21):1633-1637.
9. Domchek SM, Weber BL. Clinical management of BRCA1 and BRCA2 mutation carriers. *Oncogene*. 2006;25(43):5825-5831.
10. Rebbeck TR, Lynch HT, Neuhausen SL, et al. Prophylactic oophorectomy in carriers of BRCA1 or BRCA2 mutations. *N Engl J Med*. 2002;346(21):1616-1622.
11. Isakoff SJ, Mayer EL, He L, et al. TBCRC009: a multicenter phase II clinical trial of platinum monotherapy with biomarker assessment in metastatic triple-negative breast cancer. *J Clin Oncol*. 2015;33(17):1902-1909.
12. Ledermann J, Harter P, Gourley C, et al. Olaparib maintenance therapy in patients with platinum-sensitive relapsed serous ovarian cancer: a preplanned retrospective analysis of outcomes by BRCA status in a randomised phase 2 trial. *Lancet Oncol*. 2014;15(8):852-861.
13. Horak P, Frohling S, Glimm H. Integrating next-generation sequencing into clinical oncology: strategies, promises and pitfalls. *ESMO Open*. 2016;1(5):e000094.
14. Riahi A, Kharrat M, Ghourabi ME, et al. Mutation spectrum and prevalence of BRCA1 and BRCA2 genes in patients with familial and early-onset breast/ovarian cancer from Tunisia. *Clin Genet*. 2015;87(2):155-160.
15. Maistro S, Teixeira N, Encinas G, et al. Germline mutations in BRCA1 and BRCA2 in epithelial ovarian cancer patients in Brazil. *BMC Cancer*. 2016;16(1):934.
16. Zhao Q, Yang J, Li L, Cao D, Yu M, Shen K. Germline and somatic mutations in homologous recombination genes among Chinese ovarian cancer patients detected using next-generation sequencing. *J Gynecol Oncol*. 2017;28(4):e39.
17. Millan Catalan O, Campos-Parra AD, Vazquez-Romo R, et al. A multi-center study of BRCA1 and BRCA2 germline mutations in Mexican-Mestizo breast cancer families reveals mutations unreported in Latin American population. *Cancers (Basel)*. 2019;11(9):1246.
18. Samorodnitsky E, Jewell BM, Hagopian R, et al. Evaluation of hybridization capture versus amplicon-based methods for whole-exome sequencing. *Hum Mutat*. 2015;36(9):903-914.
19. Dacheva D, Dodova R, Popov I, et al. Validation of an NGS approach for diagnostic BRCA1/BRCA2 mutation testing. *Mol Diagn Ther*. 2015;19(2):119-130.
20. D'Argenio V, Esposito MV, Telese A, et al. The molecular analysis of BRCA1 and BRCA2: next-generation sequencing supersedes conventional approaches. *Clin Chim Acta*. 2015;446:221-225.
21. Park J, Jang W, Chae H, Kim Y, Chi HY, Kim M. Comparison of targeted next-generation and sanger sequencing for the BRCA1 and BRCA2 mutation screening. *Ann Lab Med*. 2016;36(2):197-201.
22. Capone GL, Putignano AL, Trujillo Saavedra S, et al. Evaluation of a next-generation sequencing assay for BRCA1 and BRCA2 mutation detection. *J Mol Diagn*. 2018;20(1):87-94.
23. Yoo J, Lee GD, Kim JH, et al. Clinical validity of next-generation sequencing multi-gene panel testing for detecting pathogenic variants in patients with hereditary breast-ovarian cancer syndrome. *Ann Lab Med*. 2020;40(2):148-154.
24. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American college of medical genetics and genomics and the association for molecular pathology. *Genet Med*. 2015;17(5):405-424.
25. Claes K, Poppe B, Coene I, Paepe AD, Messiaen L. BRCA1 and BRCA2 germline mutation spectrum and frequencies in Belgian breast/ovarian cancer families. *Br J Cancer*. 2004;90(6):1244-1251.
26. Brohet RM, Velthuizen ME, Hogervorst FB, et al. Breast and ovarian cancer risks in a large series of clinically ascertained families with a high proportion of BRCA1 and BRCA2 Dutch founder mutations. *J Med Genet*. 2014;51(2):98-107.
27. Clark SL, Rodriguez AM, Snyder RR, Hankins GDV, Boehning D. Structure-function of the tumor suppressor BRCA1. *Comput Struct Biotechnol J*. 2012;1(1):e201204005.
28. Strom CM, Rivera S, Elzinga C, et al. Development and validation of a next-generation sequencing assay for BRCA1 and BRCA2 variants for the clinical laboratory. *PLoS One*. 2015;10(8):e0136419.
29. Kim D-H, Chae H, Jo I, et al. Identification of large genomic rearrangement of BRCA1/2 in high risk patients in Korea. *BMC Med Genet*. 2017;18(1):38.
30. Kang E, Seong MW, Park SK, et al. The prevalence and spectrum of BRCA1 and BRCA2 mutations in Korean population: recent update

of the Korean Hereditary Breast Cancer (KOHBRA) study. *Breast Cancer Res Treat*. 2015;151(1):157-168.

31. Cho JY, Cho DY, Ahn SH, et al. Large genomic rearrangement of BRCA1 and BRCA2 genes in familial breast cancer patients in Korea. *Fam Cancer*. 2014;13(2):205-211.

32. Seong MW, Cho SI, Noh DY, et al. Low contribution of BRCA1/2 genomic rearrangement to high-risk breast cancer in the Korean population. *Fam Cancer*. 2009;8(4):505-508.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.