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Utility of Next-Generation Sequencing Panel Including Hereditary Breast and Ovarian Cancer-Related Genes for Pathogenic Variant Detection

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Hereditary breast and ovarian cancer syndrome (HBOC) is an inherited disorder associated with a higher than normal risk of breast and ovarian cancer. Most HBOC patients possess certain pathogenic variants (PVs) in *BRCA1/2* genes. However, studies have indicated that HBOC patients may also have PVs in other cancer-related genes. Therefore, we analyzed variants in *BRCA1/2* and other hereditary cancer-related genes in suspected HBOC patients using the multi-gene next-generation sequencing (NGS) panel method. We enrolled a total of 148 patients with cancers related to HBOC including breast, ovarian, primary peritoneal, prostate, and fallopian tube cancer. The 48 multi-gene NGS assay was applied to all samples, and multiplex ligation-dependent probe amplification (MLPA) and direct sequencing were used to confirm variants in *BRCA1/2* and Lynch syndrome-related genes. We identified 17 PVs or likely PVs in 148 participants (11.5%), with PVs in *BRCA1/2* detected in 7 patients (4.7%). We found PVs other than *BRCA1/2* in 10 patients through the NGS panel and MLPA (7.1%). Apart from *BRCA1/2*, the genes in which PVs were detected included *RAD51D*, *MLH1*, *MSH2*, and *MSH6*. The NGS method shows significant potential in diagnosing and treating suspected HBOC patients, particularly those who test negative for *BRCA1/2* genes.

Keywords: Genes, BRCA1; Genes, BRCA2; Hereditary breast and ovarian cancer syndrome; High-throughput nucleotide sequencing

Introduction

Hereditary breast and ovarian cancer syndrome (HBOC) is characterized by an elevated risk of developing breast, ovarian, and other types of cancer [1]. HBOC represents 5% to 10% of all breast cancer cases and is strongly associated with the *BRCA1/2* gene [2,3]. *BRCA1/2* are caretaker tumor-suppressor genes that facilitate DNA break repair through homologous recombination (HR) to maintain genomic stability [4]. Pathogenic variants (PVs) in *BRCA1/2* are the most common causes of HBOC [2,3]. Traditionally, molecular testing for these genes was conducted through Sanger sequencing, and Korean HBOC studies have thoroughly investigated the *BRCA1/2* penetrance rate and HBOC incidence among high-risk groups [5-7]. However, as PVs of *BRCA1/2* are found in approximately 20% of suspected HBOC patients [8], the potential presence of cancer-related genes beyond *BRCA1/2* has been suggested.

Fewer than 30% of patients exhibiting familial breast cancer (FBC) characteristics possess PVs of high-penetrance genes [9]. High-penetrance genes other than *BRCA1/2* such as *CDH1*, *PALB2*, *PTEN*, *STK11*, and *TP53* have been

detected in 5% of FBC cases, and an additional 5% of cases reported moderate-penetrance genes including *ATM*, *BARD1*, *BRIP1*, *CHEK2*, *EPCAM*, *MLH1*, *MSH2*, *MSH6*, *PMS2*, *RAD51C*, and *RAD51D* [10,11]. The advent of next-generation sequencing (NGS) has revealed the causative genes of HBOC and facilitated the simultaneous sequencing of multiple genes. Numerous studies have assessed the clinical utility of comprehensive genetic tests, including NGS, in diagnosing breast and ovarian cancer [12,13].

Notably, simultaneous multi-gene NGS tests, which include hereditary cancer-related genes, have proven particularly beneficial for patients who are clinically suspected of HBOC but tested negative for *BRCA1/2*. Individuals carrying PVs in high-penetrance genes may also be at an increased risk of developing other cancer types. As the inheritance mode of these syndromic cancers is mainly autosomal dominant, differentiating these disorders is crucial for accurate diagnosis and implications for family testing [11]. Additionally, the identification of PVs in certain HR-related genes can assist in therapy, such as the application of poly ADP-ribose polymerase (PARP) inhibitors, as well as informing decisions about preventative surgery [14].

Therefore, we applied an NGS panel to test patients with HBOC-related cancer, to identify genetic abnormalities that could cause HBOC in addition to *BRCA1/2*. Through this approach, we sought to uncover deleterious PVs in patients who had negative results for *BRCA1/2*, thereby facilitating a more precise diagnosis.

Methods

Study population

This study included a total of 148 patients diagnosed with HBOC-related cancers base on National Comprehensive Cancer Network guidelines, including breast, ovarian, primary peritoneal, prostate, and fallopian tube cancer, who visited the Keimyung University Dongsan Hospital for genetic testing between January 2020 and July 2022. Participant information was gathered through genetic counseling and medical record reviews. All participants provided written informed consent. The Keimyung University Dongsan Hospital Institutional Review Board in Daegu, Korea, approved this study (approval no. 2023-07-007).

DNA extraction, library enrichment, and NGS

Peripheral blood samples were collected into ethylenediaminetetraacetic acid tubes. Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen). Library enrichment was performed using extracted DNA and the Ion Chef System (Thermo Fisher Scientific), which automatically generates libraries from 10 ng of DNA per sample using 2 premixed pools of 1,187 primers with the Oncomine custom assay and Ion AmpliSeq Chef Solution (Thermo Fisher Scientific). The prepared libraries were sequenced on the Ion S5 XL Sequencer using an Ion 530 chip. Our NGS panel was a multi-gene panel that included the coding sequence and intron-exon boundaries of the coding exon from hereditary cancer-related genes (*ABRAXAS1*, *APC*, *ATM*, *ATR*, *AXIN2*, *BARD1*, *BLM*, *BMPR1A*, *BRCA1*, *BRCA2*, *BRIP1*, *CDH1*, *CDKN2A*, *CFTR*, *CHEK2*, *EPCAM*, *FANCC*, *FANCG*, *FH*, *GALNT12*, *GEN1*, *GREM1*, *MEN1*, *MLH1*, *MRE11*, *MSH2*, *MSH3*, *MSH6*, *MUTYH*, *NF1*, *NBN*, *NTHL1*, *PALB2*, *PALLD*, *PMS2*, *POLD1*, *POLE*, *PRSS1*, *PTEN*, *RAD51C*, *RAD51D*, *RET*, *RPS20*, *SMAD4*, *SPINK1*, *STK11*, *TP53*, and *VH*). We analyzed FASTQ format files using the Torrent Mapping Alignment Program aligner implemented in the Torrent Suite (Thermo Fisher Scientific). The process of single-nucleotide variant (SNV) calling to generate variant call format files was performed using the Torrent Variant Caller plug-in (Thermo Fisher Scientific).

Annotation and genetic variant classification

We used the Ion Reporter software (Thermo Fisher Scientific) for SNV annotation and analysis. The detected variants were classified according to the American College of Medical Genetics and Genomics guidelines and the QCI Interpret program (Qiagen) into the following categories: pathogenic (PV), likely pathogenic (LPV), variant of unknown significance (VUS), likely benign, or benign [15]. We considered pathogenic and likely PVs significant.

Confirmatory test using Sanger sequencing and multiplex ligation-dependent probe amplification analysis (MLPA)

We confirmed NGS-detected pathogenic and likely PVs using Sanger sequencing. We performed direct sequencing of the involved exon or intronic region of PVs on a 3500xL DNA Analyzer with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem). We analyzed the data using Sequencher 5.0 (GeneCodes Corporation).

We conducted MLPA in patients who tested negative in the NGS panel. We screened for copy number variation (CNV) in *BRCA1* and *BRCA2* genes using the SALSA P002 and P045 kits and CNV in *MLH1*, *MSH2*, *MSH6* genes using the SALSA P003-D1 *MLH1/MSH2* and P072-D1 *MSH6-MUTYH* kits

(MRC-Holland). We performed MLPA as described previously [16]. We conducted fragment analysis using Coffalyser.Net (MRC-Holland). We detected CNVs when the height ratio of the polymerase chain reaction-derived fluorescence peaks was either <0.7 or >1.4. For any relevant exon that could lead to a false-positive signal, we performed Sanger sequencing of the probe binding and ligation site.

Results

Of the 148 participants, 145 were female and 3 were male. There were 107 patients with ovarian cancer, 33 with breast cancer, 1 with tubal cancer, 3 with prostate cancer, and 1 with primary peritoneal cancer. Additionally, 3 patients had double cancer diagnoses; 1 with ovarian and stomach cancer, 1 with ovarian and lung cancer, and another with breast and colorectal cancer. The mean age for female patients was 57, ranging from 32 to 91 years, and the mean age for male patients was 72 years, ranging from 64 to 78 years.

Through comprehensive testing, we identified 17 PVs or LPVs in 148 patients (11.5%). In total, 109 patients had only VUSs, and 22 patients did not display any variants. The clinical characteristics and type of variant of the participants are listed in Table 1.

PVs or LPVs in *BRCA1/2* were identified in 7 of the 148 patients (4.7%). We detected PVs of the *RAD51D* gene in 6 patients, 5 of whom had the same variant (chr17:33434458, NG_031858.1, c.270_271dup). Four other patients showed PVs or LPVs in Lynch syndrome (LS)-related genes, including *MLH1*, *MSH2*, and *MSH6*. Two patients had PVs in the *MSH2* gene, 1 of which was a CNV detected using the MLPA method. Table 2 lists the clinical characteristics and detailed PV results.

Table 1. Patient characteristics

Patient	Count (n)
Total	148
Sex	
Female	145
Male	3
Cancer type	
Ovarian	107
Breast	33
Prostate	3
Primary peritoneal	1
Tubal	1
Ovarian and stomach	1
Ovarian and lung	1
Breast and colorectal	1
Variant type	
PV or LPV	17
VUS	109
None	22

PV, pathogenic variant; LPV, likely pathogenic variant; VUS, variant of unknown significance.

Discussion

In this study, we conducted a multi-gene NGS panel to evaluate its clinical utility in HBOC patients, particularly those with negative results for *BRCA1/2* genes.

BRCA1/2 PVs were identified in 4.7% of HBOC patients, a rate lower than previously reported by the Korean Hereditary Breast Cancer (KOHBRA) large-cohort study (15.73%) [6] and the Korean Ovarian Cancer patient study (16.5%) [7]. Our study included both breast and ovarian cancer patients, as well as those with other *BRCA1/2*-related cancers. However, as we had a significantly higher number of ovarian cancer patients at the start of this study and included patients regardless of their family history of HBOC-related cancer (a crucial factor in hereditary cancer), the detection rate was expected to be lower.

Excluding *BRCA1/2*-positive patients, 10 patients tested positive, accounting for 7.1% of the sample. Comparatively, Tung et al. [17] reported that 2.9% of 377 patients without *BRCA1/2* PVs had PVs in breast or ovarian-related genes. Other studies testing Korean breast and/or ovarian cancer patients negative for *BRCA1/2* demonstrated positive rates of 7.5% and 3.3% for PVs in genes that cause HBOC, respectively [18,19]. More NGS studies on Korean HBOC patients are needed to expected to show more accurate frequency of pathological mutations in HBOC-related genes without *BRCA1/2* negative patients.

Of particular interest, the PV of the *RAD51D* gene was identified in 6 patients, 5 of whom had the same variant. The *RAD51D* gene, along with other genes such as *BRIP1* and *RAD51C*, encodes proteins that interact with *BRCA1/2* to aid DNA repair [20]. The prevalence of mutations in this gene ranges from 0.31% to 0.6% [20-22] in ovarian cancer patients, with estimated tubo-ovarian cancer odds ratios for *RAD51D* PV carriers between 6-12% [22-25]. One study showed that *RAD51D* PVs were detected in 1.7% (13/781) of ovarian cancer patients in China and c. 270_271dup mutation was the most common mutation which was found in 7 patients (53.8%, 7/13) [26]. Other study in China also presented that

Table 2. Clinical characteristics and genetic information of patients with pathogenic variants

Patient no.	Sex	Age (yr)	Diagnosis	Gene	Nucleotide	Amino acid
1	F	78	Tubal cancer	*RAD51D*	c.270_271dup	p.Lys91Ilefs*13
9	F	66	Ovarian cancer	*MLH1*	c.1684del	p.Gln562Argfs*29
24	F	61	Ovarian cancer	*RAD51D*	c.270_271dup	p.Lys91Ilefs*13
25	F	51	Ovarian cancer	*RAD51D*	c.270_271dup	p.Lys91Ilefs*13
30	F	47	Ovarian cancer	*MSH6*	c.2731C>T	p.Arg911Ter
34	F	57	Ovarian cancer	*BRCA2*	c.6724_6725del	p.Asp2242Phefs*2
35	F	51	Ovarian cancer	*RAD51D*	c.270_271dup	p.Lys91Ilefs*13
36	F	47	Breast cancer	*BRCA1*	c.1205del	p.Glu402Glyfs*8
				BRCA2	c.7480C>T	p.Arg2494Ter
51	F	78	Ovarian cancer	*BRCA2*	c.3096_3110delinsT	p.Lys1032Asnfs*5
53	F	52	Ovarian cancer	*RAD51D*	c.270_271dup	p. Lys91Ilefs*13
54	F	55	Ovarian cancer	*BRCA1*	c.5467+1G>A	
55	F	54	Breast cancer	*MSH2*	Exon 3 deletion	
57	F	53	Ovarian cancer	*MSH2*	c.187del	p.Val63Ter
89	F	79	Breast cancer	*BRCA2*	c.7480C>T	p.Arg2494Ter
99	F	49	Ovarian cancer	*BRCA1*	c.390C>A	p.Tyr130Ter
114	F	45	Ovarian cancer	*BRCA1*	c.302-2A>C	
134	F	51	Ovarian cancer	*RAD51D*	c.694C>T	p.Arg232Ter

Patients with breast and ovarian cancer simultaneously were counted in duplicate to include each cancer type. Ovarian cancer includes primary peritoneal and fallopian tube cancers.
F, female; M, male.

most common mutation in 450 epithelial ovarian cancer patients other than *BRCA1/2* was *RAD51D* c. 270_271dup (n = 8) [27]. Because of the rarity of study of *RAD51D* PV, there is very limited information of the clinical characteristics and detailed mechanism of oncogenesis. Including our study, the two studies described above suggest that the role of *RAD51D* germline mutation of ovarian cancer may play a role differently for other races, resulting in a high frequency of occurrence in Asian, therefore, more comprehensive study about *RAD51D* germline mutation in HBOC patients are helpful to understand the role of *RAD51D* genes. And as an HR-related gene, patients with germline *RAD51D* PVs can also benefit from PARP inhibitor therapy [14,28]. Some studies have recommended risk-reducing surgery for *RAD51D* mutation carriers, especially those aged 40-50 years [29]. These findings support the suggestion that multi-gene tests, including HR-related genes, should be preferentially offered to patients with negative *BRCA1/2* results to provide appropriate treatment opportunities.

LS is an autosomal dominant inherited disease triggered by a germline mutation in one of the DNA mismatch repair (MMR) genes, such as *MLH1*, *MSH2*, *MSH6*, and *PMS2* [30,31]. MMR gene mutations can lead to colorectal cancer and potentially other cancers, including gastric, pancreatic, and ovarian cancer [32]. In this study, we detected 4 PVs in MMR genes; 3 patients with ovarian cancer had pathogenic SNVs in *MLH1*, *MSH2*, and *MSH6*, whereas 1 patient with breast cancer exhibited a deletion of exon 3 in *MSH2*. One study showed that 51% of females had an endometrial or ovarian cancer diagnosed first in a total of 117 females with dual primary colorectal/gynecologic cancers fulfilled Amsterdam criteria for LS, showing a tendency to occur earlier than colorectal cancer [33]. In this study, 2 out of 3 patients with ovarian cancer had a family history related to LS, therefore, even patients with single primary gynecological cancer need to rule out LS if they have a family history of LS-related cancer. And despite ongoing debate about whether breast cancer is part of the LS-related cancer spectrum, some cohort studies have reported increased age-specific incidence of breast cancer in LS [34,35]. In particular, this variant was a CNV detectable only through the MLPA method; therefore, if LS symptoms are strongly suspected, considering the MLPA following negative NGS test results could aid diagnosis. Despite the MLPA method is not extensively covered by the Korean medical care at present, our findings suggest that there is a need for future expansion in the insurance domain.

Our study had certain limitations. First, sufficient family history was not collected for all participants, which may have

affected the PV-positive rate of this study. Second, we did not validate all VUSs detected in our results. A more comprehensive evaluation of VUSs could influence the results.

In conclusion, the NGS panel comprising 48 HBOC-related genes demonstrated significant clinical utility in suspected HBOC patients, particularly those with negative *BRCA1/2* results. Despite the lower incidence rate of CNVs in HBOC-related genes, we recommend additional genetic tests such as MLPA, which can substantially aid precise diagnosis, contingent on the patient's clinical symptoms.

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Ethics approval

Participant information was gathered through genetic counseling and medical record reviews. All participants provided written informed consent. The Keimyung University Dongsan Hospital Institutional Review Board in Daegu, Korea, approved this study (approval no. 2023-07-007).

Conflict of interest

The authors have nothing to disclose.

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