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Next generation sequencing is a reliable tool for detecting *BRCA1/2* mutations, including large genomic rearrangements

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이 논문을 박사학위 논문으로 제출함

2021년 8월

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Acknowledgement

본 연구를 진행하고 마무리 짓기까지 많은 분들이 큰 도움을 주셨습니다. 연구의 방향성을 제시해 주시고 논문 준비 전반에 걸쳐 많은 도움을 주신 김도훈 교수님을 비롯하여, 심사과정에서 부족한 점을 보완해 주신 하정숙 교수님, 류남희 교수님께 감사드립니다. 바쁜 시간 중에도 본 논문의 심사를 위해 기꺼이 귀한 시간을 할애해 주신 강선희 교수님과 송경은 교수님께도 감사의 마음을 전달하고 싶습니다. 또한 본 연구와 관련한 실험을 할 수 있도록 검사실 장비와 자리를 마련해 주신 분자진단실의 선생님들과 의국 구성원 교수님들께도 감사의 인사를 전합니다.

이번의 연구 경험을 바탕으로, 항상 탐구하고 학문적으로 발전하는 진단검사의학과 의사가 되도록 노력하겠습니다.

마지막으로 늘 제 편에서 아낌없는 응원과 사랑을 보내주었던 저희 가족들에게도 감사의 인사를 드리고 싶습니다.

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1. Introduction

BRCA1 and *BRCA2* (*BRCA1/2*) are tumor suppressor genes that participate in DNA repair in response to DNA damage [1,2]. Deleterious germline variants in these genes can increase the risks of breast, ovarian, and several other types of cancer, including fallopian tube, primary peritoneal, prostate, and pancreatic cancer [3,4]. In breast and ovarian cancer, *BRCA1/2* mutations show especially high penetrance and cumulative cancer risks [5,6]. Therefore, comprehensive *BRCA1/2* gene analysis is important for breast and ovarian cancer patients, and their family members.

The spectrum of harmful *BRCA1/2* variants is broad, and includes small-scale mutations, such as single-nucleotide variants (SNVs), small insertions or deletions, and large genomic rearrangements (LGRs). Most of the pathogenic variants in these genes are small-scale mutations. However, LGRs are also important genetic factors in the development of cancer. A number of studies of *BRCA1/2* LGRs have been performed in several countries, and the results indicated variations in prevalence by ethnicity and country. A number of studies in several western countries have reported a higher prevalance of LGRs, 6 LGRs of 15 pathogenic *BRCA1* mutations in northern Italian breast/ovarian cancer families, 27% LGRs of the total 121 *BRCA1* mutation-positive families in Dutch population, and 16 LGRs in 19 of 53 breast/ovarian cancer patients in Portuguese [7-10]. A few *BRCA1/2* LGR studies have been conducted in Korea, and showed a relatively low prevalence compared with western countries, 1.8% LGRs in 111 Sanger-negative patients, 3.7% (3/81) LGRs of the patients who had mutations, 7% LGRs among Sanger-negative patients, and one LGR in 226 patients [11-14]. Sanger

sequencing and multiplex ligation-dependent probe amplification (MLPA) have been the gold standards to test for small-scale mutations and LGRs in *BRCA1/2*, respectively. However, since the introduction of next-generation sequencing (NGS), these techniques are no longer seen as cost-effective and rapid methods, especially in populations with a low prevalence of LGRs.

NGS has been implemented as a rapid and cost-effective *BRCA1/2* testing strategy [15,16]. The continuous evolution of NGS has facilitated the detection of LGRs, as well as small-scale mutations in a single-workflow trial. The NGS-based detection of LGRs has some advantage compared with MLPA, including not requiring the inclusion of additional control samples for each analysis, lower input concentration of DNA, and avoiding the potential for false-positive results caused by variations in the MLPA primer hybridization site.

Oncomine™ BRCA Research Assay (Thermo Fisher Scientific, Rockford, IL, USA) is a commercial NGS-based platform used in many clinical laboratories. NGS-based platforms can reduce the turnaround time for comprehensive *BRCA1/2* gene analysis, but the performance of NGS for detecting small-scale mutations and LGRs for complete *BRCA1/2* testing has not been evaluated.

In this study, the author evaluated Oncomine™ BRCA Research Assay based on the Ion Torrent S5 Platform for identification of small-scale mutations and LGRs simultaneously using a single workflow. The author compared the data acquired from NGS with the confirmatory results obtained by Sanger sequencing and MLPA. The author also evaluated the performance of this NGS-based platform for simultaneous detection of small-scale mutations and LGRs, as a comprehensive *BRCA1/2* gene testing method using different versions of NGS data analysis software.

2. Materials and Methods

2.1 Study population

A total of 258 female patients with breast, ovarian, primary peritoneal and fallopian tube cancer and family members with family history of breast/ovarian cancer, who visited Keimyung University Dongsan Hospital for genetic testing between February 2016 and March 2021, were enrolled in this study. The patients considered to have a family history of cancer were those with one or more close (first-, second-, or third-degree) blood relatives with *BRCA1/2*-associated cancers (breast, ovarian, pancreatic, or prostate cancer). Clinical data, including family histories and tumor information, were collected through pre-and post-test genetic counseling and a review of the medical records. All participants provided written informed consent. This study was approved by the Institutional Review Board of Keimyung University Dongsan Hospital, Daegu, Korea (approval number: 2021-04-029).

2-2. DNA extraction

Peripheral blood samples were collected into EDTA tubes. Genomic DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The quality and quantity of DNA samples were assessed by spectrophotometry (NanoDrop ND-100 Spectrophotometer v3.01; NanoDrop Technologies Inc., Wilmington, DE, USA).

2-3. Sanger sequencing and MLPA

Sanger sequencing of the samples of participants enrolled between February 2016 and April 2018 was performed, to detect small-scale mutations in all exons and intron regions within 20 nucleotides from the exon - intron boundary using a 3500xL DNA Analyzer with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Data were analyzed using Sequencher 5.0 software (Gene Codes Corporation, Ann Arbor, MI, USA). Starting with samples collected after April 2018, Sanger sequencing was performed for confirmation when pathogenic, likely pathogenic variants or variants of uncertain significance (VUS) were detected by NGS-based *BRCA1/2* analysis. Exon numbering and DNA sequence variant descriptions were based on NM_007294.3 and NM_000059.3, which were used as reference sequences for *BRCA1/2*.

MLPA was conducted in all participants. LGRs were screened by SALSA P002 and P045 kits, with the P087 and P077 kits used for confirmatory testing (MRC-Holland, Amsterdam, The Netherlands). MLPA was performed as described previously [14]. Coffalyser.Net (MRC-Holland) was used for fragment analysis. The height ratio of the PCR-derived fluorescence peaks was measured to quantify the amount of PCR product after normalization, and LGRs were identified when the ratio was < 0.7 or > 1.4. Sanger sequencing of the probe binding and ligation sites was conducted to detect any variants that could lead to a false-positive results.

2-4. BRCA1/2 SNV and LGR analysis using NGS

The library was prepared using the Ion Chef System (Thermo Fisher Scientific), which can automatically generate libraries from 10 ng of DNA per sample with two premixed pools of 265 primers using Oncomine™ BRCA Research Assay and an Ion AmpliSeq Chef Solutions DL8 Kit (Thermo Fisher Scientific). After clonal amplification, the prepared libraries were sequenced on an Ion S5 XL Sequencer using an Ion 520 Chip. Data in FASTQ format were analyzed using the Torrent Mapping Alignment Program aligner implemented in Torrent Suite software (Thermo Fisher Scientific). The author used the plug-in Torrent Variant Caller (Thermo Fisher Scientific) for SNV calling to generate variant call format (VCF) files and for coverage analysis. Ion Reporter™ (IR) Software (Thermo Fisher Scientific) was used for annotation and analysis of *BRCA1/2* small-scale mutations and LGRs in the Oncomine™ BRCA analysis workflow. While using Oncomine™ BRCA Research Assay, IR was updated several times, from v5.4 to the latest version of v5.16. As IR v5.4 was retired after upgrading the servers to IR v5.16, it is currently unavailable for analysis. The author compared all variations detected by each IR version and checked for differences in the results. To determine the validity of this workflow for detecting LGRs, the author calculated the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for each version.

LGRs were detected using two independent algorithms, i.e., GeneCNVs and Exon Level Deletion/Duplication (Thermo Fisher Scientific). In the case of whole gene copy number variation (CNV), the author used the GeneCNVs algorithm, in which the mean coverage of each gene was compared by the *t* test and called as CNV if there was a significant difference in the proportions of Phred scores $\geq$ 40. The Exon Level Deletion/Duplication algorithm was based on copy number, normalized using MAXgene (Thermo Fisher Scientific) to the gene with the highest

median coverage, after correction relative to the variability control information baseline (VCIB). Each detected CNV had a confidence score, which reflects how confident the software is with the call being made; a confidence score < 10 was considered no call, but single-exon CNVs required a confidence score > 18 to be considered a true call from IR v5.16. All variations analyzed using this pipeline, including small-scale mutations and LGRs, were visualized in IR software.

2-5. Statistical analysis

Statistical analysis to assess the relations between *BRCA1/2* mutational status and clinicopathological information was performed using SPSS software (version 20.0; IBM Corp., Armonk, NY, USA). The number of patients with fallopian tube and peritoneal cancer was combined with the number of patients with ovarian cancer for statistical analysis using the χ^2 test and Fisher's exact test. In all analyses, $p < 0.05$ was taken to indicate statistical significance.

3. Results

Of the 258 participants, 126 had breast cancer, 114 had ovarian cancer, 9 had primary peritoneal cancer, and 2 had fallopian tube cancer. One patient had both breast and ovarian cancer. Family members who only had family cancer history without personal cancer history were 6.

The mean age of all participants in this study was 55 years (24 - 85 years). The majority of breast cancers were of the ductal carcinoma (n = 117, 92.1%), T1 tumor (n = 72, 56.7%), and luminal (n = 87, 68.5%) subtypes. The majority of the ovarian cancer patients had serous carcinoma (n = 87, 75.7%), stage III cancer (n = 55, 47.8%), and tumor grade 3 (n = 98, 85.2%). Seventy-three patients (57.5%) with breast cancer and twenty (17.4%) with ovarian cancer had a relevant family history. The clinical characteristics of the cancer patients are listed Table 1.

Through comprehensive *BRCA1/2* gene testing, the author detected 32 pathogenic or likely pathogenic variants in 43 of 258 participants (16.6%). Twenty-four cancer patients and one family member who only had family cancer history had *BRCA1* variants, and eighteen patients had *BRCA2* variants. Five LGRs were detected, accounting for 1.9% (5/258) of all recruited participants and 9.3% (5/43) of all positive patients (Table 2).

Twelve (9.4%) breast cancer patients had pathogenic *BRCA1/2* gene variants. Among these 12 patients, 10 (83.4%) had ductal carcinoma, 7 (58.3%) had T1 tumor, and 7 (58.3%) had the luminal molecular subtype. Thirty-one (24.6%) ovarian cancer patients had pathogenic variants. Among these 31 patients, 28 (90.3%) had serous histological type, 25 (80.6%) had stage III - IV, and 31 (100%) had tumor grade 3 (Table 3).

No significant associations were observed between *BRCA1/2* mutational status and clinicopathological data.

Of the 25 patients with deleterious variants in *BRCA1*, 4 had breast cancer, 19 had ovarian cancer, and 1 had primary peritoneal cancer. Of the 18 patients with variants in *BRCA2*, 7 had breast cancer, 10 had ovarian cancer, and 1 had both breast and ovarian cancer. Three ovarian cancer patients and one patient with primary peritoneal cancer had LGR in *BRCA1*. One LGR in *BRCA1* was detected in a family member of the breast/ovarian cancer patient. All detected LGRs were exon-level deletions; two samples showed deletion of exons 1 - 2, one showed deletion of exons 1 - 13, one showed deletion of exon 23, and one showed deletion of exons 21 - 23 (Figure 1).

All pathogenic/likely pathogenic variants and VUS detected by NGS were compared with the results of confirmatory tests by Sanger sequencing and MLPA. No differences were found in small-scale variants between NGS and confirmatory tests.

However, the results of LGR detection were different among IR versions (Table 4). Four LGRs (samples 1 - 4) were detected by all IR versions, but one (sample 5) variant was not detected by IR v5.6. That is, all LGRs were identified by IR v5.10. The detected exon numbers of two LGRs (samples 4 and 5) were changed from IR v5.12 because of the use of transcript NM_007294.3 instead of NM_007300.3 for *BRCA1* in the exon deletion algorithm. The confidence score did not show linearity as the versions were updated. All confidence scores for each sample after IR v5.12 were identical.

The sensitivity, specificity, PPV, and NPV of this test for LGRs generally increased in the later IR versions (Table 5). The most recent version, IR v5.16, showed the best results for all indexes. Sensitivity was 100% from IR v5.10 onward. But, in IR v5.16, the specificity was

99.8 %, due to a significant decrease in false-positives.

Table 1. Clinical characteristics of the study population (n=258)

Cancer	Characteristic	Value
Breast (n=127*)	Age at diagnosis (yr)	47(27-80)
	Family history	73(57.5)
	Histologic type	
	Ductal	117(92.1)
	Lobular	3(2.4)
	Others	7(5.5)
	Tumor size	
	Tis	13(10.2)
	T1	72(56.7)
	T2	37(29.1)
	T3	2(1.6)
	T4	3(2.4)
	Molecular subtype	
	Triple-negative	29(22.8)
	Luminal	87(68.5)
	HER2+	11(8.7)
Ovarian (n=115*)	Age at diagnosis (yr)	55(24-85)
	Family history	20(17.4)
	Histologic type	
	serous	87(75.7)
	clear cell	8(7)
	mucinous	6(5.2)
	endometroid	3(2.6)
	seromucinous	1(0.8)

	others	10(8.7)
	Stage	
	Ⅰ-Ⅱ	36(31.3)
	Ⅲ-Ⅳ	79(68.7)
	Tumor grade	
	1-2	17(14.8)
	3	98(85.2)
Peritoneal (n=9)	Age at diagnosis (yr)	71(40-78)
	Family history	0
	Histologic type	
	serous	8(88.9)
	mucinous	1(11.1)
Fallopian tube (n=2)	Age at diagnosis (yr)	71.5(63-80)
	Family history	0
	Histologic type	
	serous	2(100)
Family members of cancer patients (n=6)	Age at genetic test (yr)	55(48-61)
	Family history	6(100)

Data are described as the mean (range) or number (%).

*The number of patients with both breast and ovarian cancer is counted in duplicate by each number of breast and ovarian cancer, respectively.

Abbreviation: HER2, human epidermal growth factor receptor 2.

Table 2. Pathogenic variants detected by Ion Reporter™ Software and confirmatory tests

Gene	Nucleotide	Protein	Function	Cancer type (No.)	No.
BRCA1	c.390C>A	p.Tyr130Ter	Nonsense	OC	1
	c.981_982del	p.Cyc328Ter	Nonsense	OC	1
	c.1205del	p.Glu402Glyfs*8	Frameshift	BC(1), OC(1)	2
	c.1336_1343dup	p.His448Glnfs*8	Frameshift	OC	1
	c.3059del	p.Pro1020Glnfs*4	Frameshift	OC	1
	c.3231del	p.Pro1078Glnfs*3	Frameshift	OC	1
	c.3412G>T	p.Gly1138Ter	Nonsense	OC	1
	c.3627dupA	p.Glu1210Argfs*9	Frameshift	OC(2)	2
	c.4117G>T	p.Glu1373Ter	Nonsense	OC	1
	c.5030_5033del	p.Thr1677Ilefs*2	Frameshift	OC	1
	c.5080G>T	p.Glu1694Ter	Nonsense	BC(1), OC(1)	2
	c.5266C>T	p.Gln1756Ter	Nonsense	BC	1
	c.5339T>C	p.Leu1780Pro	Synonymous	BC(1), OC(2)	3
	c.5445G>A	p.Trp1815Ter	Nonsense	OC	1
	c.5483del	p.Cys1828Leufs*6	Frameshift	OC	1
	Deletion of exon 1-2		LGR	FM(1), OC(1)	2

	Deletion of exon 2-13		LGR	PC	1
	Deletion of exon 21-23		LGR	OC	1
	Deletion of exon 23		LGR	OC	1
BRCA2	c.759delT	p.Ser253Argfs*24	Frameshift	BC	1
	c.1399A>T	p.Lys467Ter	Nonsense	BC&OC(1), OC(1)	2
	c.3599_3600del	p.Cys1200Ter	Nonsense	OC	1
	c.3744_3747del	p.Ser1248Argfs*10	Frameshift	BC	1
	c.5576_5579del	p.Ile1859Lysfs*3	Frameshift	BC(1), OC(2)	3
	c.5795_5799del	p.His1932Profs*11	Frameshift	BC	1
	c.6553del	p.Ala2185Leufs*6	Frameshift	OC(2)	2
	c.6724_6725del	p.Asp2242Phefs*2	Frameshift	OC	1
	c.7258G>T	p.Glu2420Ter	Nonsense	BC(2)	2
	c.7641del	p.Lys2547Asnfs*4	Frameshift	BC	1
	c.7480C>T	p.Arg2494Ter	Nonsense	OC(2)	2
	c.9117G>A	p.Pro3039Pro	Splice site	OC	1

Abbreviations: BC, breast cancer; FM, family member of cancer patient; LGR, large genomic rearrangement; OC, ovarian cancer; PC, primary peritoneal cancer.

Table 3. Correlation of the clinical characteristics with mutation status in breast and ovarian cancer

Cancer	Characteristic	*BRCA1/2* positive	*BRCA1/2* negative	p value
Breast (n=127*)	No.	12(9.4)	115(90.6)	
	Age at diagnosis(yr)	45(34-59)	52.7(27-80)	
	Family history	7(58.3)	65(56.5)	0.789
	Histologic type			0.584
	Ductal	10(83.4)	107(93.1)	
	Lobular	1(8.3)	2(1.7)	
	Others	1(8.3)	6(5.2)	
	Tumor size			0.405
	Tis	1(8.3)	12(10.4)	
	T1	7(58.3)	65(56.5)	
	T2	4(33.3)	33(28.7)	
	T3	0	2(1.8)	
	T4	0	3(2.6)	
	Molecular subtype			0.402
	Triple-negative	4(33.3)	25(21.7)	
	Luminal	7(58.3)	80(69.6)	
	HER2+	1(8.3)	10(8.7)	
Ovarian** (n=126*)	No.	31(24.6)	95(75.4)	
	Age at diagnosis	54(41-73)	56(24-85)	
	Family history	7(22.6)	13(13.7)	0.379
	Histologic type			
	serous	28(90.3)	69(72.6)	
	clear cell	0	8(8.4)	

mucinous	0	6(6.2)	
endometroid	0	3(3.3)	
seromucinous	0	1(1.1)	
others	3(9.7)	8(8.4)	
Stage			0.363
Ⅰ-Ⅱ	6(19.4)	30(31.6)	
Ⅲ-Ⅳ	25(80.6)	65(68.4)	
Tumor grade			0.148
1-2	0	17(17.9)	
3	31(100)	78(82.1)	

Data are described as the mean (range) or number (%).

*The number of patients with breast and ovarian cancer simultaneously is counted in duplicate by each number of breast and ovarian cancer respectively.

**Ovarian cancer includes primary peritoneal and fallopian tube cancers.

Table 4. Information of *BRCA1/2* LGRs detected by Ion Reporter™ Software by version

Sample (MLPA result)		IR v5.6	IR v5.10	IR v5.12	IR v5.14	IR v5.16
1 (Exon 1-2 deletion)	Confidence score	100	86.46	69.94	69.94	69.94
	Copy number	1	1	1	1	1
2 (Exon 1-2 deletion)	Confidence score	37.3	42.97	40.54	40.54	40.54
	Copy number	1	1	1	1	1
3 (Exon 1-13 deletion)	Confidence score	100	100	100	100	100
	Copy number	1	1	1	1	1
4 (Exon 21-23 deletion)	Confidence score	100	100	100	100	100
	Copy number	1	1	1	1	1
5 (Exon 23 deletion)	Confidence score	Not detected	26.72	39.51	39.51	39.51
	Copy number		1	1	1	1

Abbreviations: IR, Ion Reporter™; MLPA, multiplex ligation-dependent probe amplification.

Table 5. Performance validation of *BRCA1/2* LGR detection by Ion Reporter™ Software by version

		MLPA(+)	MLPA(-)	Sensitivity(%)	Specificity(%)	PPV(%)	PNV(%)
IR v5.6	Call	4	9	80	96.4	30.8	99.6
	No call	1	244				
IR v5.10	Call	5	12	100	95.2	29.4	100
	No call	0	241				
IR v5.12	Call	5	12	100	95.2	29.4	100
	No call	0	241				
IR v5.14	Call	5	10	100	96	33.3	100
	No call	0	243				
IR v5.16	Call	5	3	100	98.8	62.5	100
	No call	0	250				

Abbreviations: IR, Ion Reporter™; MLPA, multiplex ligation-dependent probe amplification; NPV, negative predictive value; PPV, positive predictive value.

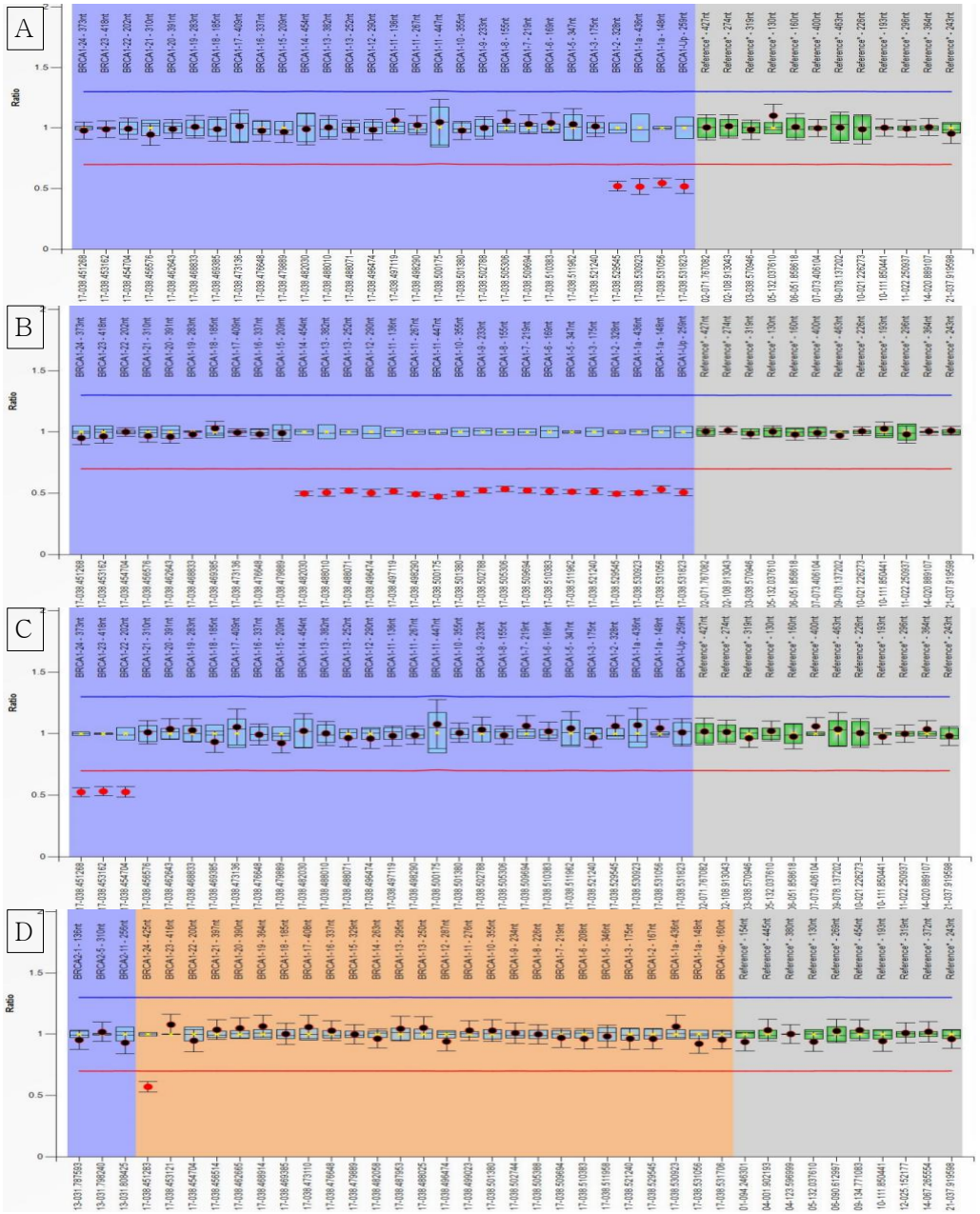

Figure. 1. MLPA results of samples with *BRCA1* LGRs in bar chart generated by Coffalyser.Net. Exons with reduced peak ratio are indicated by red dot. (A) Sample 1 with deletion of exon 1-2.

Sample 2 showed the same peak pattern with sample 1. (B) Sample 3 with deletion of exon 1-13. (C) Sample 4 with deletion of exon 21-23. (D) Sample 5 with deletion of exon 23. Exon numbering in bar charts were applied to the NCBI reference transcript NG_005905.2.

4. Discussion

The *BRCA1/2* test is extremely important for the patients of breast and ovarian cancer, because preventive treatments could be performed, and another genetic cancer risk could be managed with family members of cancer patients. Furthermore, patients with mutations in the *BRCA 1/2* can receive targeted therapy with poly-adenosine diphosphate ribose polymoerase (PARP) inhibitors. PARP inhibitors play a major role in single strand DNA repair and tumor cells with *BRCA1/2* mutations are targeted and destroyed by PARP inhibitors through a mechanism known as synthetic lethality [17,18]. PARP inhibitors increase the survival rate of advanced breast and ovarian cancer patients with *BRCA1/2* mutations [19,20], therefore, detection of mutation on this genes is highly related to the outcome of cancer patients.

But, the spectrum of *BRCA1/2* mutations is so broad that several tests are required for comprehensive analysis. Sanger sequencing and MLPA have been the gold standard tests for confirmation of detected variants for more than a decade, but these tests are both time-consuming and labor-intensive. With the introduction of NGS, a large number of laboratories have shifted from Sanger sequencing to NGS-based testing [21]. NGS-based platforms, including LGR detection, have continued to evolve, but still have several limitations [22,23], and optimal customization of the NGS pipeline at clinical laboratories to detect LGRs is a difficult process in many cases. For NGS platform without customizing LGR detection, additional MLPA process is essential when negative result in NGS, which is highly labor intensive and time-consuming. However, even if the prevalece of LGRs in Korea is low, confirmation of LGRs is nesessary, so an effective BRCA

screening strategy is needed in that sense.

The Oncomine™ BRCA Research Assay was launched in 2017 and quickly introduced into clinical laboratories with NGS-based platforms due to its convenience, including automated library preparation. This panel was originally designed to detect both small-scale mutations and LGRs, but it has not been properly evaluated after each update to the IR software. Since 2017, the IR software, which includes the Oncomine™ BRCA analysis workflow, has undergone several updates, including an updated CNV baseline, multiple bug fixes, and improved LGR detection filters. In the present study, all samples positive on MLPA were detected by the CNV filter of the IR software. Thus, this platform allows comprehensive *BRCA1/2* screening with a single workflow.

Several studies using Oncomine™ BRCA Research Assay for LGR detection have been reported [24 - 26]. The LGR identification performance of this platform was reported to be excellent, showing a high concordance rate in comparison of NGS and MLPA results, with one study reporting overall agreement of 100% between the Oncomine™ BRCA Research Assay and MLPA [24]. One study also showed 100% sensitivity, specificity, and accuracy of the CNV data analysis using NGS compared to MLPA [25]. Another study, performed in South Africa, reported that a total of eight confirmed LGRs were found, of which seven were detected by Oncomine™ BRCA Research Assay, and one LGR was only applied MLPA, not implemented NGS [26]. Likewise, this study also demonstrated the high accuracy of LGR detection of NGS. However, unlike previous studies, the author compared the results of NGS and MLPA with different IR versions for the same samples, and found that one of five positive samples showed false-negative results with IR v5.6. LGR detection by NGS requires a reliable CNV baseline, which is essential to obtain accurate results. IR has been

upgraded with updated CNV baseline data, which are required for proper normalization of NGS results to detect LGRs. In this study, the sensitivity was 100% from IR v5.10, and IR v5.16 showed the highest specificity (98.8%) by reducing the high false-positive rate observed for the previous version, because of a change in the confidence score criteria that can be a true call in single-exon CNV, which was considered a true call only when the confidence score was ≥ 18 from IR v5.16. The high sensitivity of this test is a major advantage as an LGR screening test. This workflow can accurately detect LGR-positive samples and allows confirmation tests, such as MLPA, to be applied only to suspected LGR-positive samples, thus improving the efficiency of the *BRCA1/2* gene testing process.

All LGRs detected in this study were present only in the *BRCA1* gene, similar to previous reports [27,28]. The greater number of LGRs in the *BRCA1* than *BRCA2* gene was probably due to the high content of intronic *Alu* repeat sequences in the *BRCA1* gene [29], which are involved in unequal homologous recombination and represent the major mechanism for the occurrence of LGRs.

This study had some limitations. First, LGR of *BRCA1* exon 1 was not detectable by NGS because Oncomine™ BRCA Research Assay did not cover noncoding regions. Therefore, the LGRs detected in samples 1 - 3 by NGS did not include exon 1 LGRs (Table 4). Therefore, the author confirmed *BRCA1* exon 1 deletion in each sample by MLPA. This platform required additional primers and data analysis programs for CNV detection in exon 1 of *BRCA1*, because this exon includes the core promoter of the gene, which should be included in *BRCA1/2* genetic testing to detect promoter-region deletions [30]. With the exception of *BRCA1* exon 1, the author found that this test was able to accurately identify the locations of deleted exons. Second, due to the extremely low

frequency of *BRCA2* LGRs in Korea, this present study did not include cases of *BRCA2* LGR. Further multicenter studies with larger sample sizes would be required.

Although the incidence rate of LGRs in *BRCA1/2* in Korea is low, screening tests for LGRs should be performed. A *BRCA1/2* genetic test by NGS was required to allow simultaneous analysis of small-scale mutations and LGRs. This study demonstrated that confirmation tests, such as MLPA, were required only in samples with positive LGR results on Oncomine™ BRCA Research Assay. This process could be used as an efficient and safe strategy for routine *BRCA1/2* genetic testing. In addition, this method could also facilitate rapid and accurate determination of treatment strategies, such as targeted therapies and risk-reducing surgery.

5. Summary

The spectrum of *BRCA1/2* harmful variants is broad, including single nucleotide variants (SNVs), small insertion or deletion and large genomic rearrangements (LGRs). Next generation sequencing (NGS) has been implemented as fast and cost-effective *BRCA1/2* screening strategy. The author evaluate Oncomine™ BRCA Research assay (Thermo Fisher Scientific, Rockford, IL, USA) that simultaneous detection of small-scale mutations and LGRs as comprehensive *BRCA1/2* gene test by different software version of NGS data analysis.

A total of 258 female patients with breast/ovarian (including primary peritoneal and fallopian tube) cancer or a family history of cancer. The NGS assay was implemented for all samples, and the results were compared with those of Sanger sequencing and MLPA. All small-scale variations in Sanger sequencing were detected by NGS assay.

However, the results of LGR detection were different among IR versions. Four LGRs were detected by all IR versions, but one variant was not detected by IR v5.6. The sensitivity, specificity, PPV, and NPV of this test for LGRs generally increased in the later IR versions. All LGRs were identified by IR v5.10, and the most recent version, IR v5.16, showed the best results for all indexes. Oncomine™ BRCA Research assay could be used as an efficient and safe strategy for routine *BRCA1/2* genetic testing and could help determine the treatment strategy.

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Next generation sequencing is a reliable tool for detecting *BRCA1/2* mutations, including large genomic rearrangements

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(Abstract)

Next-generation sequencing (NGS) has been implemented as a rapid and cost-effective *BRCA1/2* test strategy. The Oncomine™ BRCA Research Assay is an NGS-based tool for simultaneous detection of small-scale mutations and large genomic rearrangements (LGRs). The author evaluated this NGS assay using different versions of Ion Reporter™ (IR) software. A total of 258 patients with breast, ovarian cancer, or a family history thereof, were enrolled in the study. The NGS assay was implemented for all samples, and the results were compared with those of Sanger sequencing and MLPA. All small-scale variations in Sanger sequencing were successfully detected by NGS assay. For the detection of LGRs, this assay showed 100% sensitivity with IR v5.10,

and the latest version of the software (v5.16) showed the highest specificity. Throughout this study, NGS with an appropriately updated workflow proved reliable for comprehensive *BRCA1/2* gene testing, including LGR screening, which could facilitate efficient and accurate decision-making regarding treatment.

Large genomic rearrangements를 포함한 *BRCA1/2* 돌연변이 검출에 있어서 차세대 염기서열 분석법의 신뢰도 연구

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(초록)

차세대 염기서열 분석법(next generation sequencing, NGS)은 검사의 편의성과 경제성으로 인해 *BRCA1/2* 유전자 검사로 많이 사용된다. OncomineTM BRCA Research Assay 검사는 NGS 기반 BRCA1/2 유전자 검사로 작은 범위의 돌연변이와 large genomic rearrangement(LGR)를 동시에 검출 가능하다. 이 연구는 해당 검사 내 분석 프로그램인 Ion Reporter™(IR) Software의 개선 시점 별 결과와 확진 검사의 결과를 비교하여 수행하였다. 총 258명의 유방암, 난소암 환자 및 유방암과 난소암 가족력이 있는 가족 구성원을 대상으로 하였다. NGS는 모든 검체에 적용하였고, 그 결과를 돌연변이 확진 검사인 직접염기서열분석법과 다중결찰탐색자증폭(Multilex ligation dependent probe amplification, MLPA) 결과와 비교하였다. LGR에 있어서, IR 5.6판 이후에 민감도가 100% 도출되었고, 가장 최근 개선된 IR 5.16판에서는 가장 높은 특이도가 도출되었다. 이 연구

를 통해 계속적인 개선 과정을 거친 NGS 검사는 LGR 검출 여부를 선별할 수 있는 포괄적 *BRCA1/2* 유전자 검사로써 검사의 효율성을 높이고 치료 결정에 도움을 줄 수 있을 것으로 생각된다.