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BRCA1/2 somatic mutation detection in formalin-fixed paraffin embedded tissue by next-generation sequencing in Korean ovarian cancer patients

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ABSTRACT

Introduction: The detection of *BRCA1/2* mutations is important because PARP1 inhibitors are approved for germline and/or somatic *BRCA*-mutated advanced ovarian cancer. Next-generation sequencing (NGS) is increasingly used in clinical practice for *BRCA1/2* mutations. The purpose of this study was to consider several conditions of NGS *BRCA1/2* assay applicable to clinical laboratory tests, in particular for using formalin fixed paraffin embedded (FFPE) ovarian tissues.

Materials and methods: We selected 64 ovarian cancer patients and performed Oncomine™ BRCA assay using FFPE tissue. Effect of FFPE sample quality was analyzed by NGS quality parameters including deamination metric. Somatic variants were selected by removing germline variants of peripheral blood and interpreted as pathogenic, variants of unknown significance, and false positive.

Results: We found a positive relationship between the number of variants over the deamination metric and FFPE age ($P < 0.001$) with a cutoff values of approximately 0.7 and 60 months, respectively. When comparing NGS results with Sanger sequencing, NGS misreported 3 of 15 variants using default parameters which were corrected after changing parameters. We detected somatic variants in eight patients and classified them into pathogenic (n = 3), VUS (n = 3) and false positive (n = 2).

Conclusions: This study is important for improving *BRCA1/2* mutation detection capabilities of NGS analytical pipelines and strategy to overcome their limitations using FFPE tissue in ovarian cancer patients.

1. Introduction

BRCA1 and BRCA2 (BRCA1/2) play a critical role in double-strand DNA break repair via homologous recombination. [5] Carriers of BRCA mutations have increased risk for development of breast and ovarian cancer. A cumulative risk of breast and ovarian cancer to age 80 was 72% and 44% for *BRCA1* and 69% and 17% for *BRCA2*, respectively. [11] Inactivation of wild-type alleles of the *BRCA1* or *BRCA2* genes (second hit) in a mutation carrier is a key process of carcinogenesis leading to a homologous recombination repair (HRR) deficient tumor. The poly (adenosine diphosphate [ADP]-ribose) polymerase 1 (PARP1) also has a major role in DNA repair through base excision repair of DNA single-strand breaks. PARP1 inhibitors are "synthetic lethal" to BRCA1/2 mutations in HRR-deficient tumors. [7] Currently, PARP1 inhibitors are approved by the US Food and Drug Administration (FDA) for germline and/or somatic BRCA-mutated advanced ovarian cancer. In Korean and European, Olaparib (Lynparza) can be used in patients with somatic and germline pathogenic variants. Germline *BRCA1/2* mutations are reported in 11.5%–15.3% of non-selected ovarian cancer patients. [15,18,19] The prevalence of somatic mutations in the *BRCA1/2* genes has been reported as 0%–8.7%, a difference in part due to ethnic differences and small study numbers [1–3,7,8,12,13,16,17].

To identify BRCA mutations, Sanger sequencing on blood has been traditionally used as the gold standard. Next-generation sequencing

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(NGS) assays have been increasingly used in clinical practice, and it has been reported that BRCA NGS assays are more sensitive, have a shorter turn-around-time, and cost less per sample than conventional Sanger sequencing. [4] BRCA NGS assays with tumor DNA detects somatic variants and is recommended for management of ovarian cancer patients [7].

In this study, we evaluated *BRCA1/2* mutations in ovarian cancer tumor tissue by NGS and considered several conditions applicable to clinical laboratory tests, in particular for using formalin fixed paraffin embedded (FFPE) tissues.

2. Methods

2.1. Patients and DNA extraction

We selected 64 ovarian cancer patients through retrospective chart review from 2009 to 2016 in Seoul St. Mary's Hospital who had undergone a debulking operation and had BRCA Sanger sequencing performed on the blood. This study was approved by the Institutional Review Board of The Catholic University of Korea (KC15SISE0263). The patients' ages ranged from 18 to 76 years old (mean 51 years old). FFPE tissue slides were reviewed by a pathologist with a specialty in gynecology (A. L.). Histological diagnoses consisted of high-grade serous carcinoma (n = 44), mucinous carcinoma (n = 10), clear cell carcinoma (n = 6), and endometrioid carcinoma (n = 4). Representative tumor areas were marked on hematoxylin and eosin (H&E) stained slides based on a minimum tumor cell percent of 20%. Corresponding area of marked tumors were macro-dissected from two to five cuts of 10 μm thick unstained slices. DNA was extracted using a Maxwell® 16 FFPE Tissue Low-Elution Volume DNA Purification kit on an automated Maxwell 16 instrument (Promega GmbH, Mannheim, Germany). Nucleic acid quality and quantity was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA concentration was determined using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific), and this concentration was used for library preparation.

2.2. Tumor cell count

Each slide was scanned at low-power (x40) magnification to select fields that represent the average tumor cell percent. One microscopic photograph was obtained at a magnification of x200 using an Olympus DP72 digital camera (Olympus, Tokyo, Japan) on an Olympus BX-51 microscope. With the aid of an image processing program (Adobe Photoshop; Adobe Systems Incorporated, San Jose, CA, USA), total tumor cells and non-tumor cells including inflammatory cells, endothelial cells, and stromal cells were counted (Fig. 1). The number of counted cells ranged from 719 to 2928 (mean 1617.5), and the percent tumor cells ranged from 24.5% to 95.1% (mean 75.7%; median 78%) (Supplemental Table 1).

2.3. Identification of BRCA1/2 germline mutations

Sanger sequencing of the *BRCA1/2* genes had been performed at the Department of Laboratory Medicine according to the laboratory manual. The origin of these samples has been described in a previous publication. [9] In brief, Sanger sequencing of blood-extracted DNA was performed on all the coding exons and flanking intronic regions of *BRCA1* and *BRCA2*. PCR amplicons were bidirectionally sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Chromatograms were analyzed with Sequencher software version 5.0 (Gene Codes, Ann Arbor, MI, USA). To identify copy number variations (CNVs), MLPA (MRC-Holland, Amsterdam, Netherlands) was performed using genomic DNA isolated from fresh frozen blood of the same 64 patients.

2.4. NGS for BRCA1/2 mutations

Libraries were prepared using an Ion Chef System (Thermo Fisher Scientific) according to the manufacturer's instruction. In short, barcoded libraries were generated from 10 ng of DNA per sample using an Ion AmpliSeq™ kit for Chef DL8 (Thermo Fisher Scientific) and the Oncomine™ BRCA assay. This assay has 2 primer pools with a total of 265 primer pairs covering all exonic regions of the *BRCA1/2* genes and is optimized for FFPE tissue. The prepared libraries were clonally amplified onto Ion Sphere Particles (ISP) using emulsion PCR in an Ion Chef System (Thermo Fisher Scientific). Enriched ISPs were loaded onto Ion 520 chips accommodating up to eight tumor samples per sequencing run on a single chip. The Ion Torrent Sequencer, S5XL was used to sequence them.

The base calling and alignment of the sequences to reference genome hg19 were performed on the Ion Torrent Suite Software (Version 5.2.2). Ion Reporter software (Version 5.4) was used for variant calling and CNV analyses.

3. Results

3.1. NGS quality parameters for BRCA1/2 mutations

Various quality parameters were provided, including mapped reads, on-target read rate, average read depth, uniformity, end-to-end metrics, and strand bias (Supplement Table 2). Uniformity ranged from 90.14% to 100%, and the on-target read rate ranged from 92.82% to 96.58% (Fig. 2A). The end-to-end base coverage for samples ranged from 93.47% to 96.79%, and the end-to-end amplicon ranged from 98.11 to 99.87% for all 64 samples. Strand bias is an indicator of the similarity in the performance of the forward and reverse strands during sequencing and can be determined both as a fraction of bases and as a fraction of amplicons. For all 64 samples, the target base strand bias ranged from 0.27% to 4.46% as a fraction of bases and from 0% to 3.77% as a fraction of amplicons (Fig. 2B).

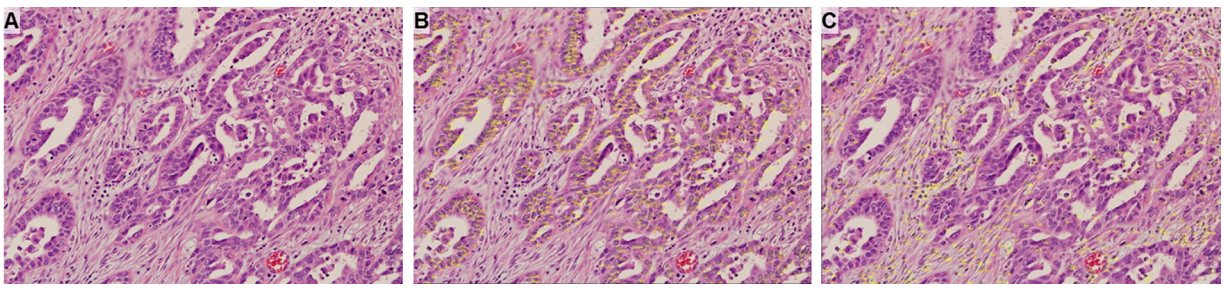

Fig. 1. Microscopic photograph was obtained at a magnification of x200. Total tumor cells and non-tumor cells including inflammatory cells, endothelial cells, and stromal cells were counted. The percent of tumor cells ranged from 24.5% to 95.1% (A, B, C).

Table 1
Somatic variants detected in tumor tissue.

Sample	Gene	DNA variants	Protein change	Allelic frequency	Deamination matric	Final interpretation
CMC1	*BRCA2*	c.4796_4814delATAATGATAAAAACCTTGT	p.Asn1599fs	16.90%	0.81	Pathogenic
CMC47	*BRCA2*	c.123_124insACCC	p.Tyr42fs	35.20%	0.52	Pathogenic
CMC51	*BRCA1*	c.1981delA	p.Arg661fs	59.74%	0.49	Pathogenic
CMC13	*BRCA2*	c.964A > C	p.Lys322Gln	19.00%	0.81	VUS
CMC31	*BRCA2*	c.9397 T > A	p.Ser3133Thr	18.07%	0.62	VUS
CMC39	*BRCA1*	c.2199 G > T	p.Glu733Asp	27.44%	0.49	VUS
		c.1334C > A	p.Ser445Tyr	22.03%		
CMC41	*BRCA2*	c.3895 G > A	p.Glu1299Lys	5.09%	0.80	False positive
		c.7455 G > A	p.(=)	5.21%		
		c.9217 G > A	p.Asp3073Asn	6.70%		
CMC22	*BRCA2*	c.2228_2229insC	p.Ser744fs	99.00%	0.55	False positive

VUS, variant of unknown significance.

3.2. Effect of FFPE sample quality on NGS for BRCA1/2 mutations

In the current study, FFPE tissues ranged from 9 to 99 months in age. We analyzed the effect of age on NGS for *BRCA1/2* mutations. There was no apparent association between DNA yields and age of the FFPE tissue (Fig. 3A). A deamination metric is calculated by measuring the frequency of the deamination (C > T plus G > A type) events in all substitution event counts. A simple linear regression fit between the number of variants and the deamination metric showed a positive relationship ($R^2 = 0.2582$, $P < 0.001$) but with a higher standard error (SE = 91.76). In addition, a linear regression fit between the number of variants and FFPE age showed a positive relationship ($R^2 = 0.2424$, $P < 0.001$, SE = 0.63). Similarly, a linear regression fit between the deamination metric and FFPE age showed a positive relationship ($R^2 = 0.6115$, $P < 0.001$, SE < 0.001). We developed a multiple linear regression model to explain this dependence and found a positive relationship between the number of variants over the deamination metric and FFPE age ($P < 0.001$) suggesting that the number of variants reported is dependent on the FFPE age as well as deamination. Fig. 3B shows the deamination metric of all 64 samples, the number of mutations reported, and FFPE age in months. There is a clear cutoff value of approximately 0.7 in the deamination metric above, which indicates that the number of reported variants exceeds the expected number. In addition, we observed that FFPE-derived DNA above 60 months of age had higher deamination as quantified by a deamination metric, which also translated to higher variant numbers.

3.3. Somatic BRCA1/2 variants in FFPE tumor tissues using NGS

We detected 15 germline pathogenic variants in the blood by Sanger sequencing using default parameters. Oncomine™ BRCA misreported two complex variants including c.5496_5506delGGTGACCCGAGinsA as c.5559 G > A (n = 2) and c.1293_1295delACTinsGA as c.1293_1295delACTinsGAG (n = 1) using the default parameters. This is most likely a discrimination error due to the complex variant present at this position. These variants were detected well when changing the parameters to the Ion Torrent Server-Torrent Suite 5.2.2 parameters. Of the 15 pathogenic variants detected in tumor samples, the reported allelic frequency (AF) of germline mutations varied from 35.4% to 92.7%. The vast majority (13/15) are well above the expected germline frequency of 50%. Somatic variants were detected in eight patients by NGS using DNA extracted from tumor FFPE (Table 1). When mutations detected in FFPE tumor tissue were identified in peripheral blood, they were considered germline mutations. Three were considered as pathogenic variants, *BRCA2* c.4796_4814delATAATGATAAAAACCTTGT (p.Asn1599fs), *BRCA2* c.123_124insACCC (p.Tyr42fs), c.123_124insACCC p.Tyr42fs, and *BRCA1* c.1981delA (p.Arg661fs). They were all frameshift mutations with 16.90%, 35.20%, and 59.74% of AF. Three variants were considered as variants of unknown significance (VUS) after reviewing mutation type, AF, deamination metric, adjacent sequences, and comparison with signals of other cases. *BRCA2* c.964A > C with 19.00% of AF detected in CMC 13 considered as a VUS because of the considerable AF and absence of same variant in other cases even though a relatively high deamination metric (0.811). In CMC 31, *BRCA2* c.9397 T > A was detected with an AF 18.07%, which was considered as a VUS after an Integrative Genomics Viewer

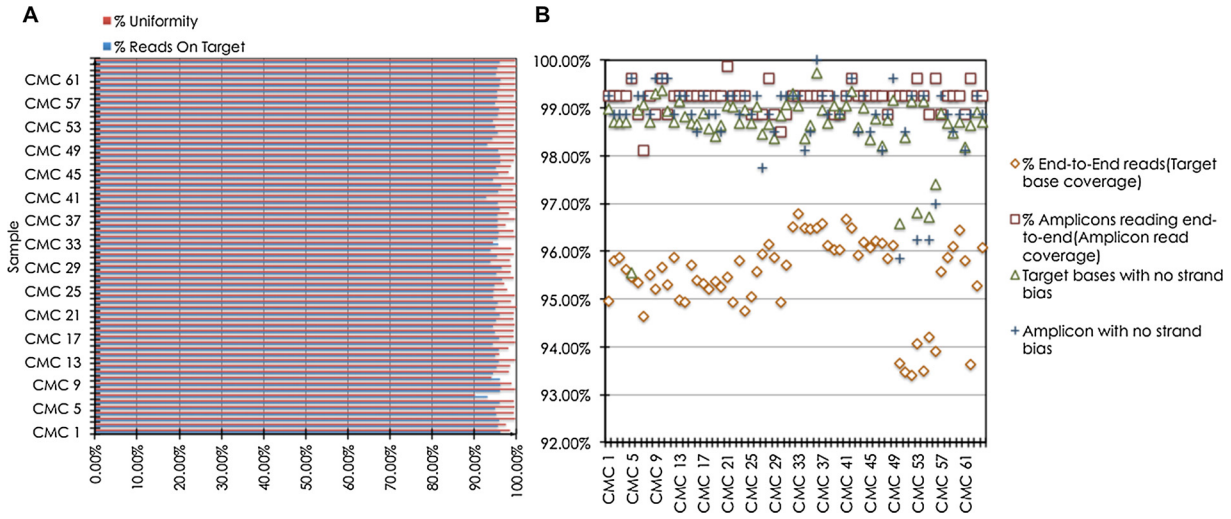

Fig. 2. Various quality parameters were provided, including mapped reads, on-target read rate, average read depth, uniformity, end-to-end metrics, and strand bias.

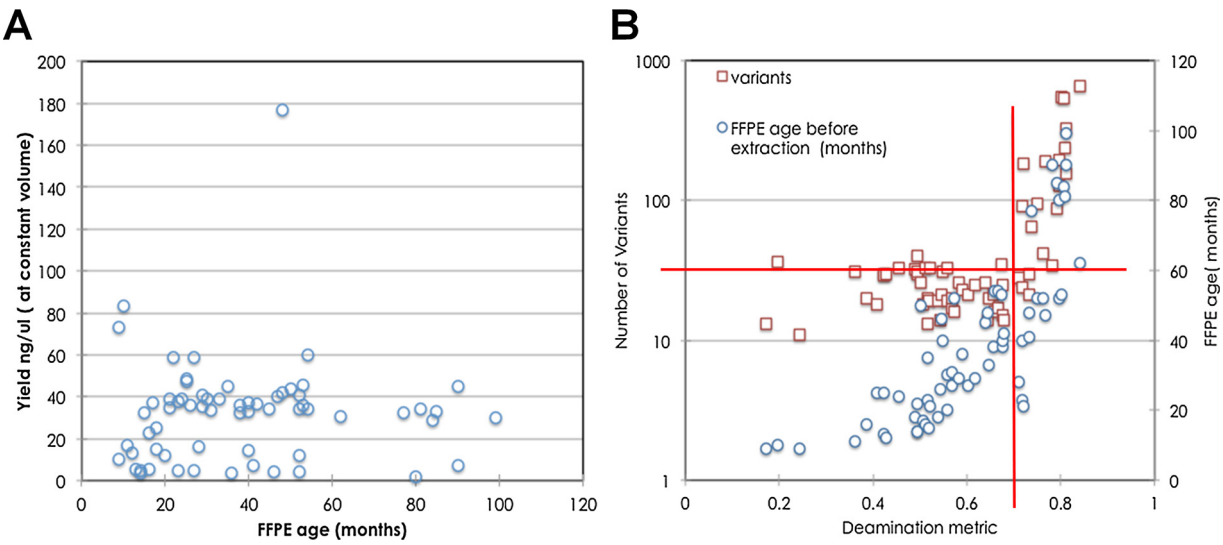

Fig. 3. There was no apparent association between DNA yields and age of the FFPE tissue (A). A deamination metric is calculated (B). Deamination metric of all 64 samples showed positive relation with the number of mutations reported, and FFPE age in months. There was a clear cutoff value of approximately 0.7 in the deamination metric.

review even though an adjacent polyA region. In CMC39, NGS detected two SNV in the *BRCA1* gene (c.2199 G > T) and the *BRCA2* gene (c.1334C > A) whose AFs were 27.44% and 22.03%, respectively. We considered these variants as neither germline (due to their absence the blood Sanger sequencing results) nor a deamination effect (deamination metric of 0.49, which is below the 0.7 cutoff). After removing the possibility of noise signals, we considered them as VUS. Variants in two cases were interpreted as false positive. In CMC41, three SNVs in the *BRCA2* gene (c.3895 G > A, c.7455 G > A, and c.9217 G > A) were detected at AFs of 5.09%, 5.21%, and 6.7%, respectively. A deamination metric of 0.80, above the 0.7 cutoff, provides certainty that this was a deamination event. An insertion with 99.00% of AF was observed in CMC22, which was considered as false positive due to strand bias.

4. Discussion

Nowadays, NGS is increasingly being utilized for multiplexing capabilities and speed while Sanger sequencing is important for assay validation and accuracy. The first consideration to apply NGS using FFPE tissue in clinical laboratory was tissue condition. We got an excellent performance of Oncomine™ BRCA assay through macro-dissection of representative ovarian tumor areas. It is known that FFPE tissues are subjected to deamination leading to nucleotide changes. [10] However, a majority of NGS-based studies routinely rely on FFPE-derived nucleic acids and fail to consider these effects on study outcomes. While FFPE tissues provide portability and long-term storage advantages, caution is needed to ensure that the integrity of the tissues does not affect results of a study. Here, we demonstrated that deamination effects are linked to FFPE age and, consequently, the number of variants found in BRCA genes. We observed that FFPE-derived DNA above 60 months of age had higher deamination as well as higher variant numbers. In addition, we got a cutoff value of 0.7 to estimate the quality of FFPE tissues. We found a clear cutoff in the deamination metric (Fig. 3B), which indicates that the number of reported variants exceeds the expected number. Studies refer to the use of Uracil-DNA Glycosylase (UDG) to eliminate deamination effects. [6] Therefore, researchers should monitor the effects of deamination consider options like UDG treatment to avoid deamination effects from older FFPE tissues.

Next consideration was to detect variants properly and to interpret them accurately. When comparing NGS results with Sanger sequencing, NGS was incomplete to detect complex variants. However, it could be corrected through upgrading of analytical parameters. In addition, CNVs, including whole exon and gene deletion, has been known to be another challenging area in NGS. Even though a “one-size-fits-all” model might not be particularly effective now, there have been continual efforts to upgrade analytical pipelines and improve the detection power of complex variants and somatic CNVs by NGS. [14] Optimization of baseline parameters and verification of the parameters are needed before applying in the clinical laboratory. To interpret detected variants, we firstly discriminated somatic variants by removing germline variants of peripheral blood. Then we classified the somatic variants into pathogenic, VUS, and false positive after reviewing mutation type, AF, deamination metric, adjacent sequences, and comparison with signals of other cases. Better analytical strategy should be prepared for accurate interpretation under a lot more data accumulation with genotype-phenotype correlation to minimize false positive and reclassify VUS into clear clinical category.

This study shows that, when using FFPE tissue for NGS, a deamination metric of less than 0.7 and a FFPE age of less than 60 months would yield reliable results. It is important for improving *BRCA1/2* mutation detection capabilities of NGS analytical pipelines and strategy to overcome their limitations using FFPE tissue in ovarian cancer patients. In addition, clinical laboratories should understand NGS technology and utilize in applications on companion diagnostics for targeted therapy.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prp.2019.152595.

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