Letter to the Editor
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Comparison of Targeted Next-Generation and Sanger Sequencing for the *BRCA1* and *BRCA2* Mutation Screening

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Dear Editor,

High-throughput and cost-effective *BRCA* genetic screening is needed for application of pharmacogenetics in personalized HBOC therapy. Banerjee *et al*. [1] suggested that poly(ADP-ribose) polymerase (PARP) inhibitors show considerable promise for the treatment of cancers with *BRCA1* or *BRCA2* mutations. Secord *et al*. [2] demonstrated that compared to the global use of PARP inhibition, the *BRCA1* and *BRCA2* test for personalized PARP inhibition treatment may represent a de facto cost-reducing strategy.

In this study, results of targeted Next-generation sequencing (NGS) by Ion Torrent Personal Genome Machine (PGM, Life Technologies, Guilford, CT, USA) were compared with those of Sanger sequencing in seven HBOC patients harboring pathogenic variant or rare variant of *BRCA1* and *BRCA2* of uncertain clinical significance.

All enrolled subjects provided written informed consent for clinical and molecular analyses, and the study protocol was approved by the institutional review board (KC15SISE0263) of The Catholic University, Seoul, Korea. Seven HBOC patients harboring pathogenic or unclassified variants of *BRCA1* and *BRCA2* as confirmed by Sanger sequencing were studied. The Ion AmpliSeq *BRCA1* and *BRCA2* Panel (Life Technologies) consisting of 167 primer pairs in three primer pair pools was used for targeted NGS analysis. Sequencing on the Ion Torrent PGM was performed by using 500 flow runs that generated approximately 200 bp reads. Torrent Variant Caller 3.4 was applied for alignment and variant detection. The variant caller parameter setting was germline PGM high stringency (Table 1). Sequence data was visually confirmed with the Integrative Genomics Viewer (IGV) and any sequence, alignment, or variant call error artifacts were discarded. Non-synonymous variants called were evaluated by using ClinVar (http://www.ncbi.nlm.nih.gov/clinvar), the BIC database (http://research.nhgri.nih.gov/bic/), and the

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Table 1. Override parameters selected for customizing hotspot calling of *BRCA1* and *BRCA2* variants in this study

Parameters	Description	Values		
		Applied	Recommended	Allowed
Minimum allele frequency	Minimum observed allele frequency required for a non-reference variant call. Lowering this value improves sensitivity and decreases specificity (and increases the ratio of false positives to true positives).	For SNPs/hotspots: 0.1 For indels: 0.1	For SNPs: 0.01-0.2 For indels: 0.05-0.2	Floats 0.0-1.0
Minimum quality	Do not call variants if the phred-scaled call quality is below this value. Lowering this value improves sensitivity and decreases specificity.	≥10	≥10	Integers ≥0
Minimum coverage	Do not call variants if the total coverage on both strands is below this value. For germline workflows, lowering coverage improves sensitivity. Lowering this value is dangerous for homo-polymer indels: this decreases specificity drastically.	For SNPs/hotspots: 6 For indels: 15	For SNPs/hotspots: 5-20 For indels: 15-30	Integers ≥0
Minimum coverage on either strand	Do not call variants if coverage on either strand is below this value. For indel calling, reducing this value improves sensitivity but at a high cost of specificity.	For SNPs/hotspots: ≥0/3 For indels: ≥5	≥3	Integers ≥0
Minimum strand bias	Do not call variants if the proportion of variant alleles from one strand if higher than this ratio.	For SNPs/ hotspots: 0.95 For indels: 0.85	0.95	Floats 0.5-1.0
Minimum relative read quality	Do not call variants if Relative Read Quality is below this threshold. A phred-scaled minimum average evidence per read or no-call.	≥6.5	≥6.5	Floats ≥0
Maximum common signal shift	Do not call variants if Common Signal Shift exceeds this threshold. If the predictions are distorted to fit the data more than this distance (relative to the size of the variant), filter this candidate position out.	0.7	0.3=30% of variant change size	Floats ≥0
Maximum reference/ variant signal shift	Do not call insertions if Reference or Variant Signal Shift exceeds this threshold. Filter observed clusters that deviate from predictions by more than this amount (relative to the size of the variant).	For ins: 0.4 For del: 0.2	0.2=20% of variant change size	Floats ≥0
hp_max_length	Maximum homopolymer length for calling indels.	10	8	Integers ≥1
downsample_to_ coverage	Reduce coverage in over-sampled locations to this value.	10,000	For germline: 400 For somatic: 2,000	Integers ≥1
utlier_probability	Prior probability that a read comes from some other distribution. Lower numbers reduce the influence of outlier observations. Higher numbers increase the influence of outliers. Empirical adjustment indicates that increasing the influence of outliers leads to more false-positives and slightly more true positives, but at a poor tradeoff.	0.01	0.005-0.01	Floats 0.0-1.0
prediction_precision	Number of pseudo-data-points suggesting our predictions match the measurements without bias.	1.0	1.0	Floats ≥0
heavy_tailed	How heavy the T-distribution tails are to allow for unusual spread in the data. This value represents the prior probability that a given read comes from some distribution other than the possibilities being evaluated. Lower values mean that more reads are forced to be assigned to one of the tested alleles, even at very poor data fit (fewer reads are thrown out, with the likely tradeoff of more false positive calls). Higher values mean that reads that are merely slightly noisy	3.0	NA	NA

Abbreviations: SNP, single nucleotide polymorphism; indel, insertion and deletion; NA, not available.

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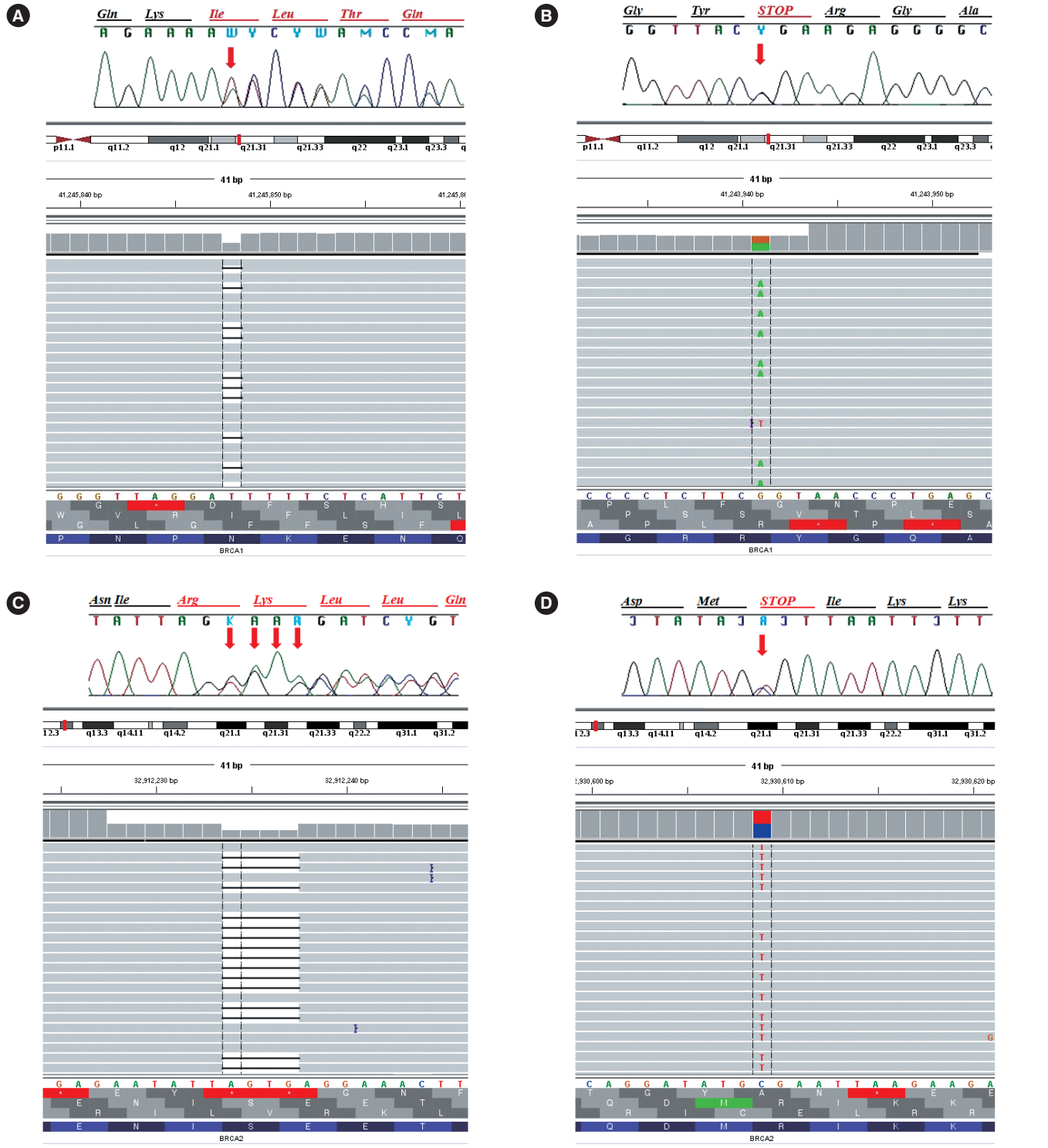

Fig. 1. Identification of deleterious mutations in *BRCA1* or *BRCA2* by Sanger sequencing and next-generation sequencing (NGS), as visualized by Sequencher Software (top) and Integrative Genomics Viewer (IGV, bottom), respectively. The deletion(s) or base change is indicated by a red arrow in Sanger sequencing and is represented by a black dashes in IGV. (A) A deletion A at position 1700 of cDNA (c.1700delA; p.Asn567Ilefs*5) of *BRCA1* in patient hereditary breast and/or ovarian cancer (HBOC)1; (B) A substitution C to T at position 3607 of cDNA (c.3607C>T; p.Arg1203*) of *BRCA1* in patients HBOC3; (C) A deletion TGAG at position 3744-3747 of cDNA (c.3744_3747delTGAG; p.Ser1248Argfs*10) of *BRCA2* in patient HBOC5; (D) A substitution C to T at position 7480 of cDNA (c.7480C>T; p.Arg2494*) of *BRCA2* in patients HBOC6.

HGMD database (http://www.hgmd.cf.ac.uk/). Minor allele frequency (MAF) was determined from the 1000 Genomes Project database (http://www.1000genomes.org/).

Technical performance of the Ion AmpliSeq *BRCA1/2* Panel showed 81% of template-positive ion sphere particle sample loading. The total read obtained was 4,541,406, with a mean read length of 137 bp. The mean sequencing depth for each region was ×494 and the average uniformity of coverage was 99%, which is percentage of target bases, covered by at least ×0.2 of the average base read depth. All variants located in all coding exons and in adjacent intronic regions of *BRCA1* and *BRCA2* identified by Sanger sequencing were detected by targeted NGS analysis. Direct sequencing of entire coding exons and flanking intronic sequences of *BRCA1* and *BRCA2* was performed on ABI 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Of seven patients, four carried deleterious *BRCA1* or *BRCA2* variants: two frameshift and two nonsense mutations. The p.Asn567Ilefs*5 and p.Arg1203* of BRCA1 have previously been reported in Turkish [3] and American [4] populations, respectively (Fig. 1A, B). The p.Ser1248Argfs*10 and p.Arg2494* of BRCA2 have been reported in German [5] and Finnish [6] populations, respectively (Fig. 1C, D). Meanwhile two *BRCA1* and one *BRCA2* out of nine non-synonymous variants are rare according to 1,000 Genome Project data (<1% population MAF). The p.Leu52Phe of BRCA1 and p.Val2109Ile of BRCA2 have been reported in the Koreans, and the p.Tyr856His of BRCA1 has been found in the Japanese populations.

Of various NGS platforms, the PGM generates DNA sequencing reads by detecting ions released when deoxynucleotide triphosphates are incorporated into a growing DNA strand on a semiconductor device [7]. In particular, it has been documented that indel errors occurring in homopolymer DNA regions significantly affect the specificity of indel detection owing to the nature of sequencing chemistry of PGM [8]. In this study, sequence "AGTG" at position 3972_3975 of *BRCA2* was given in NGS; however, the Human Genome Variation Society notation prescribes that on the forward strand it should be "TGAG" at position 3974_3977 as given in Sanger sequencing.

While the protein-truncating variants (either frameshift, nonsense, or splice) are located generally in the coding exons or the flanking intronic sequences of *BRCA* genes, potentially deleterious alterations may also reside in the noncoding intronic sequences. For example, deep intronic mutation causing activation of a cryptic exon in *BRCA2* has been reported in a French family with a history of breast cancer [9]. Thus, rare variants called with both NGS and Sanger sequencing should be considered for validation study, regardless of their location.

In conclusion, our study is a clear example that the quality of targeted NGS of a disease-specific subset of genes is equal to the quality of Sanger sequencing, and therefore it can be implemented reliably as a stand-alone diagnostic test demonstrated by Sikkema-Raddatz B *et al*. [10].

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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