Exploring the role of homologous recombination deficiency and BRCA1/2 mutations in endometrial cancer

Jonge, M.M. de

Chapter 6

Validation and implementation of *BRCA1/2* variant screening in ovarian tumor tissue

Marthe M. de Jonge, Dina Ruano, Ronald van Eijk, Nienke van der Stoep, Maartje Nielsen, Juul T. Wijnen, Natalja T. ter Haar, Astrid Baalbergen, Monique E.M.M. Bos, Marjolein J. Kagie, Maaike P.G. Vreeswijk, Katja N. Gaarenstroom, Judith R. Kroep, Vincent T.H.B.M. Smit, Tjalling Bosse, Tom van Wezel*, Christi J. van Asperen*

***Contributed equally**

The Journal of Molecular Diagnostics 2018 Sep;20(5):600-611

Abstract

BRCA1/2 variant analysis in tumor tissue could streamline the referral of patients with epithelial ovarian, fallopian tube, or primary peritoneal cancer to genetic counselors and select patients who benefit most from targeted treatment.

We investigated the sensitivity of *BRCA1/2* variant analysis in formalin-fixed, paraffin-embedded tumor tissue using a combination of next generation sequencing and copy number variant multiplex ligation-dependent probe amplification. After optimization using a training cohort of known *BRCA1/2* mutation carriers, validation was performed in a prospective cohort in which screening of *BRCA1/2* tumor DNA and leukocyte germline DNA was performed in parallel. *BRCA1* promoter hypermethylation and pedigree analysis were also performed.

In the training cohort, 45 of 46 germline *BRCA1/*2 variants were detected (sensitivity 98%). In the prospective cohort (*n*=62), all six germline variants were identified (sensitivity 100%), together with five somatic *BRCA1/2* variants and eight cases with *BRCA1* promoter hypermethylation. In four *BRCA1/2* variant-negative patients, surveillance or prophylactic management options were offered on the basis of positive family histories.

We conclude that *BRCA1/2* formalin-fixed, paraffin-embedded tumor tissue analysis reliably detects *BRCA1/*2 variants. When taking family history of *BRCA1/2* variant-negative patients into account, tumor *BRCA1/2* variant screening allows more efficient selection of epithelial ovarian cancer patients for genetic counseling and simultaneously selects patients who benefit most from targeted treatment.

Introduction

Germline *BRCA1/*2 pathogenic variants confer elevated lifetime risks for epithelial ovarian cancer (EOC), and especially for high-grade serous ovarian, fallopian tube and primary peritoneal cancers (HGSCs).[1-3] Analysis of 489 HGSCs by The Cancer Genome Atlas Research Network demonstrated that germline *BRCA1/2* variants, somatic *BRCA1/2* variants, and epigenetic silencing of *BRCA1* via promoter hypermethylation are frequent events, found in approximately 16%, 7% and 11% of cases, respectively.[4] Other studies reported comparable rates of *BRCA1/2* defects.[1, 3, 5-8]

The high prevalence of pathogenic germline *BRCA1/*2 variants in EOC patients led to the generally accepted recommendation that all women diagnosed with EOC should receive genetic counseling and be offered genetic testing, with some slight differences observed between countries.[9, 10] In the Netherlands, *BRCA1/2* variant screening is recommended for every EOC patient, irrespective of family history, age, and histologic subtype.[10]

BRCA1 and BRCA2 have multiple roles in maintaining genome integrity and are crucial for high-fidelity repair of DNA double strand breaks via homologous recombination-mediated repair.[11, 12] *BRCA1/2*-deficient tumors show specific genomic aberrations associated with this homologous recombination repair deficiency.[13-15] The platinum sensitivity frequently observed in HGSC is thought to be related to the underlying homologous recombination repair deficiency, because homologous recombination repair is involved in the repair of DNA damage induced by these agents.[13, 16, 17] Another group of drugs that exploit the presence of homologous recombination repair deficiency in tumor cells are the poly (ADPribose) polymerase (PARP) inhibitors. By increasing the burden on homologous recombination repair, these drugs induce synthetic lethality in tumor cells with acquired homologous recombination repair deficiency.[11, 18]

Multiple studies have shown that PARP inhibitors improve progression-free survival (PFS) in platinum-sensitive, recurrent EOC.[19-23] Although recent studies also reported a significantly longer PFS of patients with relapsed platinum-sensitive *BRCA1/2* wild-type HGSC receiving niraparib[20] or olaparib[19] compared with placebo treatment, most of the PFS benefit was observed for patients with pathogenic *BRCA1/2* variants. Therefore, identification of patients with either a somatic or a germline *BRCA1/2* variant would significantly improve the selection of patients who benefit most from PARP inhibition.[19, 20, 23]

Although pathogenic germline *BRCA1/2* variants are relatively common in EOC patients, most (approximately 85%) do not have a *BRCA1/2* variant. Referring all women with EOC for genetic counseling is therefore inefficient and causes unnecessary distress. This problem could be overcome by the integration of a reliable tumor screening test in the care pathway of

ovarian cancer patients. A test for genetic variants in *BRCA1/2* should be capable of detecting both germline and somatic variants using tumor DNA derived from formalin-fixed paraffin-embedded (FFPE) tissue. Initial use of a tumor DNA test, followed by referral of only those patients with a *BRCA1/2* variant (somatic or germline) for genetic counseling would avoid an estimated 80% of referrals.

The analysis of *BRCA1/2* in low-quality, highly-fragmented FFPE-derived tumor DNA is technically challenging because *BRCA1/2* are both large genes with a wide mutation spectrum.[24-28] Several studies, mainly using high-quality blood-derived DNA, have shown that next generation sequencing (NGS) can reliably detect *BRCA1/*2 variants.[25, 29-31] Studies analyzing the performance of NGS in FFPE-derived DNA have shown promising results[25, 32-34] but none of the studies simultaneously analyzed high-quality blood-derived DNA in a prospective setting.

The aim of this study was to investigate the performance of *BRCA1/2* variant analysis in DNA isolated from FFPE tumor tissue in comparison with sequencing of leukocyte DNA (currently the gold standard in *BRCA1/2* variant screening). On the basis of the results, we recommend integrating tumor screening within the care pathway of ovarian cancer patients.

Material and methods

Tissue sample and patient selection

Training cohort

The 50 patients in the retrospective training cohort were collected as follows. First, 67 patients were randomly selected who fulfilled the following selection criteria: previously identified germline *BRCA1/2* pathogenic variants at the Laboratory for Diagnostic Genome Analysis of the Leiden University Medical Center, and breast or gynecologic malignancy. From this cohort, 33 samples were selected by expert clinical molecular geneticists (J.T.W., N.v.d.S.) for pathogenic variants that were potentially challenging to detect, including deletions, insertions, and variants in flanking introns and homopolymer regions. An additional 17 cases with pathogenic germline variants were randomly selected (not based on type of variant) to reach a total of 50 cases (Figure 1A).

COBRA cohort

For the prospective clinical implementation of *BRCA1/2* screening in ovarian tumor tissue (COBRA) cohort, women were recruited in seven participating hospitals in the southwestern region of the Netherlands from February 2016 to June 2017. Women with (a history of) EOC and not previously screened for germline *BRCA1/2* variants, were eligible for inclusion. The cohort was enriched for HGSCs. After inclusion, leukocyte DNA was used for routine

germline analysis at the Department of Clinical Genetics. Simultaneously, FFPE tumor tissue blocks were collected for parallel tumor *BRCA1/2* screening at the Department of Pathology, thus allowing detection of both somatic and germline variants (Figure 1B). The study was approved by the medical ethics committee of the Leiden University Medical Center (reference number: P16.009). Sixty-six women gave signed informed consent and were included. Routine germline *BRCA1/2* screening and tumor *BRCA1/2* screening were requested simultaneously, either directly by the treating physician (gynecologist or medical oncologist) or by the clinical geneticist.

Histopathology slides from all cases were revised by an expert gynecopathologist (T.B.) in line with the most recent (2014) World Health Organization classification system.

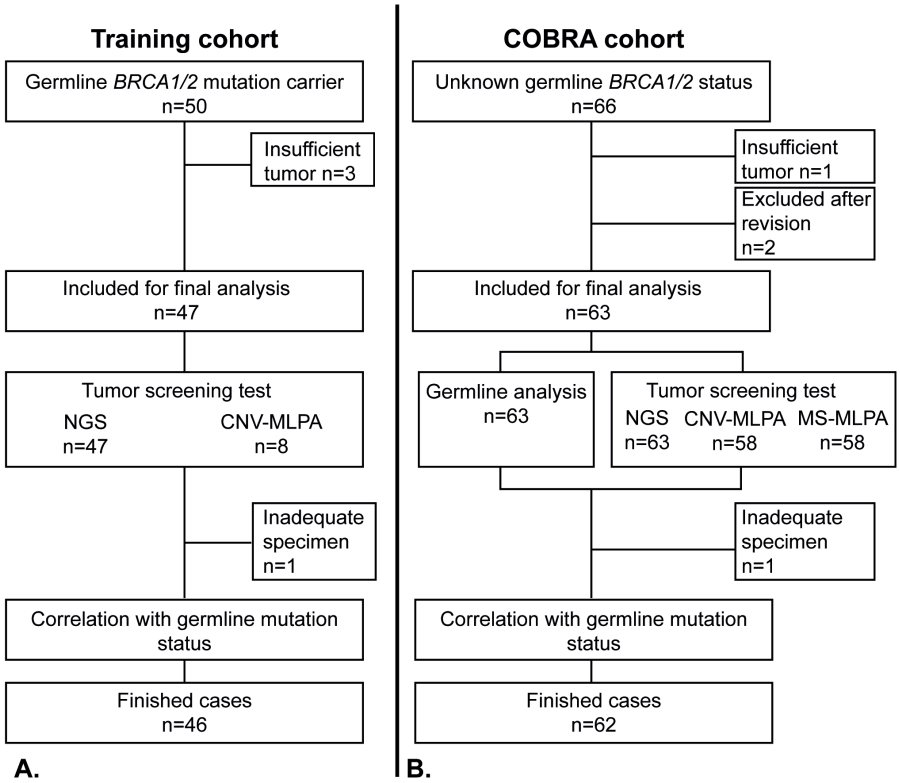


Figure 1. Schematic overview of cohort selection. A: Training cohort. Copy number variant-multiplex ligation-dependent probe amplication (CNV-MLPA) was performed only for cases in which no variant was automatically identified via the software. Of the 33 cases selected for variants that were potentially more challenging to detect, two had insufficient tumor tissue for analysis. **B:** Clinical implementation of *BRCA1/2* screening on ovarian tumor tissue (COBRA) cohort. MS, methylation specific; NGS, next-generation sequencing.

Family history

Pedigrees including first-, second- and third-degree relatives were constructed on the basis of questionnaires. The pedigrees were evaluated by expert clinical geneticists (C.J.v.A., M.N.) for tumor types and age of onset. All family histories of *BRCA1/2*-negative cases were classified on the basis of the presence or absence of an indication for extra surveillance or management options for first-degree relatives, according to current national guidelines.

DNA isolation

Tumor DNA was isolated from FFPE blocks from routine diagnostics. In most cases the tumor tissue underwent at least overnight fixation in formalin. For isolation, either three 0.6-mm tissue cores or the microdissected tumor areas from five 10-µm tissue sections was used. For the purposes of optimization, DNA from paired normal FFPE tissue was isolated and analyzed for a subset of cases in both the training cohort and COBRA cohort. The mean tumor percentage was 61% (range, 30%-90%) for the training cohort, and 65% (range, 10%-95%) for the COBRA cohort. For NGS and methylation-specific multiplex ligation-dependent probe amplification (MLPA), DNA was isolated using the automated Tissue Preparation System (Siemens Healthcare Diagnostics, Erlangen, Germany) as described previously.[35] For copy number variant (CNV) MLPA, crude DNA was manually isolated using overnight proteinase K digestion. FFPE tissue cores did not undergo deparaffinization. For microdissected samples, deparaffinization in xylene was performed, followed by rehydration through a graded ethanol series and staining with haematoxylin. Also, 20 µl of 20% chelex was added during overnight proteinase-K digestion. After overnight incubation in a heat block at 56°C, samples were heated for 10 minutes at 99°C and centrifuged at 13,000 x *g* at 4°C, after which the chelex was removed from the microdissected samples. DNA was quantified using the Qubit dsDNA HS Assay Kit, according to manufacturer's instructions (Qubit 2.0 Fluorometer; Life Technologies, Carlsbad, CA).

Next generation sequencing

BRCA1 and *BRCA2* AmpliSeq NGS libraries were prepared using the Oncomine BRCA Research panel (ThermoFisher Scientific, Waltham, MA, USA), according to the manufacturer's protocol. The panel contains 265 amplicons and covers 100% of the coding sequences of *BRCA1* and *BRCA2*, and it also includes flanking intronic sequences (average, 64 bases in 5' and 3' direction). Insert sizes (ie, the amplicon minus the primers) range from 65 to 138 bp. NGS libraries were equimolary pooled to 60 pMol/L, and the final library pool was loaded on an Ion PI chip (ThermoFisher Scientific) using an Ion Chef instrument (ThermoFisher Scientific). Sequencing was performed in an Ion Proton system (ThermoFisher Scientific).

Multiplex ligation-dependent probe amplification

CNV-MLPA was performed using the SALSA MLPA probemix P002 BRCA1 (MRC-Holland, Amsterdam, the Netherlands) on approximately 37.5 ng of DNA in a 20-µl reaction,

according to manufacturer's protocol, with small adaptations. Briefly, the SALSA probe mix and MLPA buffer were added to a solution containing approximately 37.5 ng of DNA and the mix was denatured for 10 minutes at 95°C, followed by hybridization at 60°C for 16 to 20 hours. Next, for ligation the Master mix (ligase buffer A, ligase buffer B and Ligase-65 enzyme) was added at 54°C and samples were heated for 20 minutes at 54°C, followed by 5 minutes at 98°C. The PCR master mix (including SALSA primer mix and SALSA polymerase) was then added, and the following PCR reaction was performed for 35 cycles: 30 seconds at 95°C, 30 seconds at 60°C and 60 seconds at 72°C, followed by incubation for 20 minutes at 72°C. For the training cohort, CNV-MLPA was only performed for cases in which no variant was identified via NGS data analysis. In the COBRA cohort, CNV-MLPA was performed in all cases for which sufficient tumor tissue was available.

Methylation-specific MLPA using the SALSA MLPA ME001 tumor suppressor mix (MRC-Holland) was performed, according to the manufacturer's protocol, with some adaptations. After denaturation of approximately 75 ng of DNA for 5 minutes at 98°C, the SALSA probe mix and MLPA buffer were added and samples were incubated for 1 minute at 98°C, followed by hybridization at 60°C for 16 to 20 hours. Then, ligase buffer A was added at room temperature, and the samples were heated for 2 minutes at 48°C. Samples were then split and ligated for 30 minutes at 48°C (ligase buffer B and Ligase-65 enzyme, with or without the addition of HhaI enzyme), followed by heating for 5 minutes at 98°C. After the master mix was added (SALSA primer mix and SALSA polymerase), a PCR reaction was performed for 35 cycles (30 seconds at 95°C, 30 seconds at 60°C and 60 seconds at 72°C), followed by incubation for 20 minutes at 72°C. Methylation-specific MLPA was performed for all cases from the COBRA-cohort with a [DNA] >7 ng/µl. MLPA data were analyzed using Coffalyser.Net software (MRC-Holland).

For both tests, the ABI 3130 genetic analyzer (Applied Biosystems, Foster City, CA) was used for separation of the products by electrophoresis.

Data analysis

The unaligned bam files generated by the proton sequencer were mapped against the human reference genome (GRCh37/hg19) using the TMAP 5.0.7 software with default parameters (https://github.com/iontorrent/TS, last accessed March 6, 2018). Subsequent variant calling was done using the Ion Torrent specific caller, Torrent Variant Caller 5.0.2 (Thermo Fisher Scientific), using the recommended somatic variant caller parameter for the BRCA Oncomine Panel. Briefly, variants were called with a minimum allele frequency threshold of 3.5% and a read depth of at least 100. Strand bias and proximity to a homopolymer region were also used to minimize false positives.

Integrative Genomics Viewer was used for visual inspection of the detected variants. Variants were imported into a local Genetic Assistant database (Geneticist Assistant, Version: 1.4.5;

SoftGenetics, State Collega, PA) which assigns functional prediction, conservation scores, and disease-associated information to each variant. This information is then used to assign pathogenicity to a variant, and the next time the variant is observed, the same pathogenicity is automatically attributed to the observed variant. Variant annotation was based on the NM_007294.3 and the NM_000059.3 transcripts to *BRCA1* and *BRCA2,* respectively.

Data interpretation

Variants were categorized by five-tier pathogenicity status [class 1, benign; class 2, likely benign; class 3, variant of unknown significance (VUS); class 4, likely pathogenic; class 5, pathogenic).[36]

For the training cohort, FFPE-isolated DNA was analyzed at the pathology department (Leiden University Medical Center). Although all cases were known to carry a class 4 or 5 *BRCA1* or *BRCA2* variant, it was not known which germline variant was present in the samples at the time of analysis. All variants identified were later compared with the previously identified germline variant (Figure 1A). For the COBRA cohort, the *BRCA1/2* tumor screening (at the Pathology Department of the Leiden University Medical Center) was performed concurrently with, but independently of, routine leukocyte germline screening (at the Department of Clinical Genetics, Leiden University Medical Center). On completion, the class 3, 4, and 5 variants identified in tumor DNA were compared with the results of the germline analysis (Figure 1B).

Loss of heterozygosity (LOH) of *BRCA1/2* was determined by comparing the variant allele frequency (VAF) of heterozygous SNPs and, when present, the VAF of the *BRCA1/*2 variant in tumor and normal tissue. LOH was considered present when the tumor cell percentage was >20%, the germline *BRCA1/2* variant allele frequency was >60% and/or at least two informative (heterozygous) single nucleotide variants (SNVs) showed a VAF ≤ 0.4 or ≥ 0.6. LOH was considered inconclusive when the tumor cell percentage was <20% or when only one informative SNV was present. LOH was considered absent when the germline *BRCA1/2* variant VAF was ≤ 0.6 and/or at least two informative (heterozygous) SNVs showed a VAF between 0.4 and 0.6, unless a clear difference in VAF of the SNV and/or variant could be observed between the normal DNA sample and the tumor DNA sample. LOH results were manually curated (T.v.W., R.v.E.), taking the tumor cell percentage and the VAF of the SNV or variant into account. SNVs were annotated in an in-house database (geneticist assistant).

Quality control

Sample quality was evaluated by an experienced molecular biologist (T.v.W., R.v.E.). Samples with a low coverage, a high number of low-frequency variants, or a high proportion of C:G>T:A transitions (ie, artifacts caused by formalin fixation)[26, 37] were excluded from further analysis. However, an unequivocal class 3, 4 or 5 variant identified in a poor quality sample

was considered sufficient for analysis. For the training cohort, a patient was only excluded from the final analysis if both the tumor DNA sample and the normal DNA sample failed the quality control.

Statistics

IBM SPSS software version 23.0 (IBM Corp., Armonk, NY) was used for statistical analysis. A one-way ANOVA analysis of variance was used to compare age distributions, and the Mann-Whitney U-test was used for comparison of the age of the tissue blocks. The association between histotype and *BRCA1/2* defects was tested using a two-sided Fisher's exact test. $P \leq 0.05$ was considered significant.

Results

Training cohort

Of the 50 cases in the training cohort, three were excluded because no tumor tissue was available in the archives. For the remaining 47 patients, matching normal tissue DNA was analyzed in 42 cases. Forty-six patients could be included in the final analysis because either the tumor (42/47) or the paired normal (40/42) tissue sample was sequenced with sufficient quality (Figure 1A), hence mutation status was determined on normal FFPE tissue only for four cases. One case was excluded from the analysis because sequencing results for both the tumor and the normal DNA were of insufficient quality. Tissue blocks used for DNA isolation were significantly older for the samples that failed the quality control (n=7; median, 2003; range, 1994-2014) compared to the samples that passed quality control (n=82; median, 2008; range, 1986-2015; $P < 0.05$). The median coverage per amplicon of the samples included in the final analysis is visualized in supplemental Figure S1. All 265 amplicons had a median coverage of at least 100 reads. Per sample, 98% of the amplicons (range 51.3% to 100%) were covered with a sequencing depth of at least 100 reads. Sample R27 (normal FFPE DNA) was an outlier, with only 51.3% of amplicons covered by >100 reads and 10 amplicons that completely failed. Nevertheless, a *BRCA1* variant was clearly detected, and the sample was, therefore, considered to be of sufficient quality for analysis (supplemental Table S1).

Variant analysis

The germline variants found in the 46 cases included in the final analysis are listed in Table 1. In 38 of the 46 cases (83%), a variant (SNV, small insertion or deletion) was detected during initial analysis. The *BRCA1/2* variants could be identified in both the normal and tumor DNA for all samples in which both were analyzed. All germline variants were covered by at least 100 reads and 76% of the variants had a coverage of >1000 reads.

Table 1. Germline variants in the training cohort

ID	Gene	c.DNA Change*	Amino acid change†	T%	VAF tumor	VAF normal	LOH	Histology
R31‡	BRCA1	c.68dupA	p.Cys24fs	40	0.83	0.48	Yes	EEC
R12	BRCA1	c.34C>T	p.Gln12*	70	0.96	0.43	Yes	HGSC
R35‡	BRCA1	c.81-6T>A	p.?	80	0.92	0.53	Yes	HGSC
R49	BRCA1	c.181T>G	p.Cys61Gly	70	0.81	n.a.	Yes	EEC
R11	BRCA1	c.181T>G	p.Cys61Gly	70	0.89	0.51	Yes	HGSC
R19	BRCA1	c.213-12A>G	p.?	40	0.70	0.51	Yes	EEC
R28‡	BRCA1	c.213-12A>G	p.?	65	0.74	0.56	Yes	Breast-NST
R20	BRCA1	c.[594-2A>C;c.641A>G]§	p.?	35	0,57 & 0,61	0,46 & 0,47	n.a.	LGSC
R3‡	BRCA1	c.1292dupT	p.Leu431fs	70	0.77	n.a.	Yes	HGSC
R39‡	BRCA1	c.2019delA	p.Glu673fs	60	0.73	0.45	Yes	Breast-Metaplastic
R34‡	BRCA1	c.2197_2201delGAGAA	p.Glu733fs	60	n.a. ||	0.51	n.a.	Breast-NST
R2‡	BRCA1	c.3436_3439delTGTT	p.Cys1146fs	55	0.73	0.55	Yes	Breast-ILC
R32‡	BRCA1	c.3481_3491delGAAGATACTAG	p.Glu1161fs	80	0.70¶	n.a.	Yes	HGSC
R25‡	BRCA1	c.3485delA	p.Asp1162fs	40	0.61	0.47	Yes	HGSC
R47‡	BRCA1	c.3820dupG	p.Val1274fs	80	0.97	0.47	Yes	Breast-NST
R44‡	BRCA1	c.4035delA	p.Glu1346fs	40	0.58	0.48	Not detected	HGSC
R7	BRCA1	c.4327C>T	p.Arg1443*	80	0.94	0.48	Yes	USC
R14	BRCA1	c.4327C>T	p.Arg1443	50	0.73	0.52	Yes	HGSC
R17	BRCA1	c.4327C>T	p.Arg1443	70	0.84	0.46	Yes	HGSC
R4‡	BRCA1	c.4483delA	p.Arg1495fs	60	0.53	0.51	Not detected	Breast-NST
R9	BRCA1	c.5177_5180delGAAA	p.Arg1726fs	90	n.a.¶	0.48	Yes**	HGSC
R27‡	BRCA1	c.5177_5180delGAAA	p.Arg1726fs	70	0.92	0.54	Yes	Ovarian-Mixed††
R18	BRCA1	c.5266dupC	p.Gln1756fs	75	0.99	0.49	Yes	EOC
R37‡	BRCA1	c.5266dupC	p.Gln1756fs	50	0.80	0.50	Yes	Breast-NST
R5‡	BRCA2	c.658_659delGT	p.Val220fs	60	0.47	0.43	Not detected	Breast-NST
R48	BRCA2	c.771_775delTCAAA	p.Asn257fs	80	0.81	0.56	Yes	Breast-NST
R43‡	BRCA2	c.1147delA	p.Ile383fs	50	0.84	n.a.	Yes	Breast-NST
R46‡	BRCA2	c.1147delA	p.Ile383fs	60	0.71	0.52	Yes	HGSC
R38‡	BRCA2	c.1899_1900insTT	p.Ala634fs	60	0.60	0.49	Yes	Breast-Mixed‡‡
R22‡	BRCA2	c.3599_3600delGT	p.Cys1200*	30	0.63	0.41	n.a.	Breast-NST
R24‡	BRCA2	c.4284dupT	p.Gln1429fs	70	§§	§§	Yes	Breast-NST

Continue

Continued

ID	Gene	c.DNA Change*	Amino acid change†	T%	VAF tumor	VAF normal	LOH	Histology
R33‡	BRCA2	c.5213_5216delCTTA	p.Thr1738fs	80	0.90	n.a.||	Yes	OCS
R10	BRCA2	c.5286T>A	p.Tyr1762*	80	0.71	0.62	Yes	HGSC
R8	BRCA2	c.5682C>G	p.Tyr1894*	60	0.91	0.56	Yes	HGSC
R29‡	BRCA2	c.5946delT	p.Ser1982fs	60	0.86	0.52	Yes	Breast-NST
R21‡	BRCA2	c.6270_6271delTA	p.His2090fs	40	0.75	0.51	Yes	OSC||||
R45‡	BRCA2	c.6275_6276delTT	p.Leu2092fs	70	0.79	0.51	Yes	Breast-NST
R42‡	BRCA2	c.6361_6362delGA	p.Glu2121fs	55	0.88	0.47	Yes	HGSC
R23‡	BRCA2	c.6816_6817delAA	p.Gly2274fs	70	n.a.||	0.38	n.a.	HGSC
R1‡	BRCA2	c.9099_9100delTC¶¶	p.Gln3034fs	50	0.69	0.30	Yes	Breast-NST
R36‡	BRCA2	c.9295_9301delAATTTAC	p.Asn3099fs	60	0.69¶|***	0.48¶|***	Yes	HGSC
CNV-MLPA								
R50	BRCA1	Deletion exon 8 and 9		85	n.a.||	NAP	n.a.	OCS
R15	BRCA1	Deletion exon 22		60	NAP	NAP	n.a.	HGSC
R40‡	BRCA1	Deletion exon 22		30	NAP	n.a.	Yes	HGSC
R26‡	BRCA1	c.5503_5564del	p.Arg1835fs	30	NAP	NAP	Yes	Breast-NST
R41‡	BRCA1	c.5503_5564del	p.Arg1835fs	35	NAP	NAP	Yes	Breast-NST

*Reference sequences: NM_007294.3 for *BRCA1* and NM_000059.3 for *BRCA2.* †NP_009225.1 for BRCA1 and NP_000059.3 for BRCA2. ‡Selected by expert clinical molecular geneticists for variants potentially more challenging to detect, including deletions, insertions, variants in flanking introns and in homopolymer regions. §Reclassified as a variant of uncertain significance. ||quality control failed. ¶Automatically identified after adjustment of the alignment settings. **Amplification of one of the primer pools failed; LOH based on SNVs identified in the succeeded primer pool. ††Clear cell carcinoma-endometrioid carcinoma. ‡‡NST-mucinous. §§Not detected; duplication in homopolymeric region. ||||Grading not reliable because of previous treatment. ¶¶Because of noise at the border of an 8-base pair adenine stretch, the deletion was automatically classified as delACT, but was later manually curated. ***Detected with prior knowledge of the position of the deletion.

Abbreviations; CNV-MLPA, copy number variant-multiplex ligation-dependent probe amplification; EEC, endometrioid endometrial carcinoma; EOC, endometrioid ovarian carcinoma; HGSC, high-grade ovarian, fallopian tube and primary peritoneal cancer; ID, identification; ILC, invasive lobular carcinoma; LGSC, low-grade serous carcinoma; LOH, loss of heterozygosity; NST, invasive carcinoma of no special type; n.a.,not analyzed/not analysable; NAP, not applicable, Nucl., nucleotide; OCS, ovarian carcinosarcoma; OSC, ovarian serous carcinoma; T%, tumor percentage; USC, uterine serous carcinoma; VAF, variant allele frequency.

Deletions and duplications

To detect exon deletions and duplications in *BRCA1*, CNV-MLPA was performed for the eight samples in the training cohort in which no variant was initially detected by the pipeline [either using tumor DNA (n=4), normal DNA (n=2) or both (n=2)]. This resulted in the detection of two germline deletions of exon 22 (R15 and R40), one germline deletion of exons 8 and 9 (R50) and two 62-basepair deletions in exon 24 [c.5503_5564del62, p.Arg1835Thrfs*24 (R26, R41)].

Visual inspection of the sequencing reads in Integrative Genomics Viewer for the remaining three samples revealed an 11-bp deletion (*BRCA1***;** c.3481_3491delGAAGATACTAG) and a 7-bp deletion (*BRCA2*; c.9295_9301delAATTTAC) in samples R32 and R36, respectively. Both deletions were situated at the end of a PCR amplicon, with only a few base pairs left on the short side, resulting in misalignment of the reads. Adjustment of the alignment settings improved the alignment of the reads resulting in automatic identification of both deletions (Supplemental Figure S2).

In sample R24, a known *BRCA2* variant could not be identified. The patient carried a germline duplication (c.4284dupT) in a homopolymer stretch of six thymidines. The duplication could not be identified because of sequencing artifacts present at homopolymer regions (Supplemental Figure S3).

Loss of Heterozygosity

LOH of the wild-type allele was observed in 37 cases (Table 1), whereas three cases did not show LOH. In the remaining six cases, the presence of LOH could not be determined with certainty because of a lack of informative SNPs and/or failure of sequencing of tumor DNA. Of the 16 HGSCs in which LOH could be determined, all but one showed LOH [15/16 (94%)].

Prospective COBRA cohort

In total, 66 women were recruited to participate in the prospective phase of the study (Figure 1B). Four cases (6%) were excluded from the final analysis for the following reasons:

Table 2. COBRA cohort characteristics

	Total cohort	No *BRCA1/2* defect	*BRCA1/2* variant	*BRCA1* promoter hypermethylation	*P* value
Total, *n* (%)	62 (100)	43 (100)	11 (100)	8 (100)	
Age in years, mean (range)	64 (47-89)	66 (47-89)	62 (50-69)	62 (56-71)	0.3
Tumor Type					
HGSC, *n* (%)	54 (87)	35 (81)	11 (100)	8 (100)	0.093*
Non-HGSC, n (%)†	8 (13)	8 (19)	0 (0)	0 (0)	

*The prevalence of HGSC and non-HGSC was compared between women with and without *BRCA1/2* defects.
†The non-HGSC consisted of two low-grade serous carcinomas, two endometrioid ovarian carcinomas, three ovarian clear cell carcinomas, and one ovarian carcinosarcoma.
Abbreviations; COBRA, clinical implementation of *BRCA1/2* screening on ovarian tumor tissue; HGSC, high-grade ovarian, fallopian tube, and primary peritoneal carcinoma.

insufficient tumor tissue available (n=1), quality control of tumor failed (n=1) or no ovarian malignancy after histologic revision (n=2, one metastatic endometrial cancer and one ovarian serous borderline tumor). The characteristics of the COBRA cohort are summarized in Table 2. Fifty-four patients (87%) were diagnosed with HGSC and eight patients (13%) were diagnosed with other histologic subtypes of EOC.

Of the 62 cases included in the final analysis, matched normal FFPE-derived DNA was analyzed for 37 (60%), of which four failed quality control (Supplementary Table S1).

Variant analysis was performed on FFPE cytology material for three samples, two obtained from cytocentrifuged effusions [pleural fluid (P10) and ascites (P60)], and one obtained from a lymph node puncture (P64). All produced data of sufficient quality.

Variant analysis

In total, 11 class 3, 4, or 5 *BRCA1/2* variants were identified in the tumors of 62 EOC patients (Table 3). The 10 detected variants by NGS comprised seven *BRCA1* variants, including three VUSs, and three *BRCA2* variants, including one VUS. One genomic deletion of *BRCA1* exon 22 was detected by CNV-MLPA. For six of the mutated cases in which a variant was detected by NGS, matching normal FFPE-derived DNA was analyzed, five of which produced good quality data. In one case (P30), the variant was also detected in normal FFPE material, suggesting a germline origin. The variants in P11, P14, P52 and P39 were likely somatic, given their absence in the matched normal DNA samples.

Results were compared with leukocyte germline DNA, with findings summarized in Table 3. In the leukocyte DNA, four germline *BRCA1* variants and two germline *BRCA2* variants were detected, all of which were also detected in tumor DNA, resulting in a 100% concordance in the detection of germline variants between the tumor DNA and leukocyte DNA. The remaining four *BRCA1* variants (including two VUSs) and one *BRCA2* variant were somatic variants because they were not detected in the germline DNA. No germline variants were detected in the remaining 51 samples without a *BRCA1/2* variant in tumor DNA.

BRCA1 promoter hypermethylation

With possible future clinical relevance in mind, *BRCA1* promoter hypermethylation was also analyzed in the tumors. *BRCA1* promoter hypermethylation was found in 8 of 57 (14%) cases that had sufficient tumor DNA available for methylation-specific MLPA. None of these cases had a concurrent pathogenic *BRCA1/2* variant.

All 19 *BRCA1/2* defects (germline variants, somatic variants and hypermethylated cases) were detected in patients with HGSC. There was no significant difference in age distribution between women with a *BRCA1/2* variant, with *BRCA1* promoter hypermethylation or lacking a

Table 3. *BRCA1/2* defects in the COBRA cohort

ID	Histology	Gene	cDNA change*,†	Amino acid change‡	T%	VAF tumor	VAF normal	LOH wild-type allele
Germline variants								
p18	HGSC	BRCA1	c.1881C>G§	p.Val627=	70	0,80	n.a.	yes
p32	HGSC	BRCA1	c.2685_2686delAA	p.Pro897fs	85	0,98	n.a.	yes
p56	HGSC	BRCA1	c.5277+1G>A	p.?	80	0.74	n.a.	yes
p30	HGSC	BRCA2	c.4576dupA	p.Thr1526fs	80	0,97	0,48	yes
p62	HGSC	BRCA2	c.5117A>C§	p.Asn1706Thr	80	0.54	n.a.	No
CNV-MLPA, germline								
p41	HGSC	BRCA1	Deletion exon 22	p.?	30	NAP	NAP||	yes
Somatic variants								
p24	HGSC	BRCA1	c.3718C>T	p.Gln1240*	80	0,76	Not present	yes
p11	HGSC	BRCA1	c.3858_3861delTGAG	p.Ser1286fs	70	0,56	Not present	yes
p52¶,**	HGSC	BRCA1	c.4868C>G§	p.Ala1623Gly	40	0.37	Not present	Yes††
p39	HGSC	BRCA1	c.5366C>T§	p.Ala1789Val	95	0.65	Not present	uncertain
p12	HGSC	BRCA2	c.209_210delCT	p.Ser70fs	70	0,82	n.a.‡‡	yes
MS-MLPA								
p7	HGSC	BRCA1	promoter hypermethylation	p.?	80	NAP	n.a.	uncertain
p15	HGSC	BRCA1	promoter hypermethylation	p.?	35	NAP	n.a.	yes
p17	HGSC	BRCA1	promoter hypermethylation	p.?	80	NAP	n.a.	yes
p23	HGSC	BRCA1	promoter hypermethylation	p.?	85	NAP	NAP	yes
p25	HGSC	BRCA1	promoter hypermethylation	p.?	70	NAP	NAP	yes
p36	HGSC	BRCA1	promoter hypermethylation	p.?	95	NAP	NAP	yes
p58	HGSC	BRCA1	promoter hypermethylation	p.?	70	NAP	n.a.	uncertain
p59	HGSC	BRCA1	promoter hypermethylation	p.?	70	NAP	n.a.	uncertain

All variants had a coverage well above 100 reads, reaching >1000 reads in 10 of 11 cases (91%). *Only class 3 (variant of unknown significance), class-4 (likely pathogenic), and class 5 (pathogenic) variants are reported. †Reference sequences: NM_007294.3 for *BRCA1* and NM_000059.3 for *BRCA2*. ‡Reference sequences: NP_009225.1 for BRCA1 and NP_000059.3 for BRCA2. §Variant of unknown significance. ||CNV-MLPA not performed on normal DNA sample. ¶DNA concentration too low to perform MS-MLPA. **Not enough tumor to perform CNV-MLPA. ††LOH of the mutant allele. ‡‡Quality control failed.

Abbreviations; CNV-MLPA, copy number variant-multiplex ligation-dependent probe amplification; COBRA, clinical implementation of *BRCA1/2* screening on ovarian tumor tissue; HGSC, high-grade ovarian, fallopian tube and primary peritoneal cancer; ID, identification, LOH, loss of heterozygosity; MS-MLPA, methylation specific-multiplex ligation-dependent probe amplification; n.a: not analyzed/not analyzable; NAP, not applicable; QCF, quality control failed; T%, tumor percentage; VAF, variant allele frequency.

BRCA1/2 defect (*P*=0.3) (Table 2). In cases with a *BRCA1/2* defect, LOH of the wild-type allele could be determined for 15 of 19 cases (79%). All but one case (93%) showed LOH, one of which was of the mutated allele (P52). The tumor in which no LOH was demonstrated and the one with LOH of the mutant allele both carried a VUS. No informative SNVs were present on the *BRCA1* alleles for the remaining four cases, precluding the analysis of LOH (three with

BRCA1 promoter hypermethylation and one with *BRCA1* variant). None of the six patients with a germline *BRCA1/2* variant had other malignancies in their personal history.

Comparing the frequencies of *BRCA1/2* defects in HGSC with The Cancer Genome Atlas Research Network, fewer germline mutated cases (11% versus 16%), more somatic mutated cases (9% versus 7%) and more cases with *BRCA1* promoter hypermethylation (16% versus 11%) (Supplementary Figure 4).[4]

Family history

Of the 62 patients included in the final analyses, 57 questionnaires regarding family histories were returned, which were then studied by clinical geneticists for suggestions that there was an indication for extra surveillance or management options. Regarding patients without germline *BRCA1/*2 variants, family history would have resulted in policy changes for four patients. Three patients had a positive first-degree family history for OC (P12, P52 and P59), and one patient was suspect for Lynch syndrome (i.e. fulfilled the Bethesda criteria; P55). In families with two cases of EOC but no germline variant, the ovarian cancer risk for first-degree female family members is >10%, a level at which prophylactic surgery should be considered.[38] The patient with a positive first-degree family history for colon cancer <50 years of age had a prior clear cell renal cell carcinoma but no personal history for colon cancer or endometrial cancer. Immunohistochemical staining for mismatch repair proteins did not show abnormalities, making Lynch syndrome unlikely. Nevertheless, because the family fulfilled the familial colorectal cancer criteria, advice for 5-yearly screening of the colon was given.[39]

Discussion

The aim of this study was to evaluate the reliability of *BRCA1/2* variant analysis on FFPE-derived tumor DNA, using a tumor test consisting of semiconductor sequencing with an amplicon-based *BRCA1/*2 panel combined with CNV-MLPA for *BRCA1.* During optimization of the tumor test on the training cohort, 45 of 46 variants were detected, representing a sensitivity of 98% despite enrichment for challenging variants. During prospective validation in the COBRA cohort, all six germline *BRCA1/2* variants in tumor DNA were identified (sensitivity of 100%), together with the identification of an additional five somatic *BRCA1/2* variants and eight cases with *BRCA1* promoter hypermethylation. These results show that *BRCA1/2* variants can be reliably detected in FFPE-derived DNA. In the COBRA cohort, referral based on a positive tumor *BRCA1/*2 variant screening test result may have reduced the referral rate of EOC patients to a clinical geneticist by approximately 80%.

The recent approval of the PARP inhibitors niraparib (US Food and Drug Administration, March 2017; European Medicines Agency, November 2017) and olaparib (US Food and Drug

Administration, August 2017) as maintenance treatment for platinum-sensitive relapsed HGSC regardless of *BRCA1/2* mutation status may undermine the necessity for tumor testing to detect somatic *BRCA1/2* variants. However, these approvals were based on studies showing treatment benefit (ie PFS) of PARP inhibitors in a highly-selected patient population (namely, those patients with platinum-sensitive recurrent HGSC who received at least two lines of platinum-based chemotherapy).[19, 20] *BRCA1/2* loss is known to confer sensitivity to platinum-based chemotherapy, and tumors with similar genomic scars without apparent *BRCA1/2* loss also show increased sensitivity to these agents.[13] Therefore, platinum sensitivity already selects tumors that probably carry DNA repair defects conferring sensitivity to PARP inhibitors. When platinum-based chemotherapy cannot be given or in the event that PARP inhibitors become indicated for adjuvant treatment in the future, this surrogate marker will not serve for patient selection and additional biomarkers will be needed. For the time being, known somatic and germline *BRCA1/2* mutation status helps in the selection of those patients who will derive the greatest treatment benefit from PARP inhibitors.[19, 20, 23] For example, in the study by Ledermann et al.,[19] compared with placebo, women carrying *BRCA1/2* variants showed longer PFS (11.2 versus 4.3 months) than women without *BRCA1/2* variants (7.4 versus 5.5 months).

Although patients with EOC have the highest *a priori* probability for germline variants in *BRCA1/2*, other germline predisposing variants such as *BRIP1, RAD51D* or *RAD51C* have been described.[3, 9] It is, therefore important that patients with a positive family history should still be referred to the clinical genetic services, independent of the result of a *BRCA1/2* tumor test. For example, in the COBRA cohort four patients without a germline *BRCA1/2* variant had a positive family history for either ovarian cancer or colon cancer, which can be an indication to screen for variants in additional genes or for relatives to consider prophylactic surgery. A more comprehensive tumor test incorporating additional genes seems feasible, so this limitation will likely be overcome in the future.

In the COBRA cohort, *BRCA1* promoter hypermethylation was observed in 14% of EOCs. Although hypermethylation is a well-known and common event in HGSC, its clinical relevance remains unclear. The presence of LOH in tumors with *BRCA1* promoter hypermethylation, in combination with the observed homologous recombination deficiency via functional analysis,[15] suggests that hypermethylation is an important driver of tumorigenesis. PARP inhibitor sensitivity is observed in breast cancer cell lines and xenograft tumors with epigenetic *BRCA1* silencing.[40, 41] However, it remains unclear whether this increased sensitivity also applies to patients with *BRCA1* hypermethylated EOC. In a recent study, *BRCA1* hypermethylation was not associated with an increased PARP inhibitor response,[42] whereas in the ARIEL2 trial a subset of *BRCA1*-methylated EOC showed a longer PFS.[23] In the absence of clear data on clinical consequences, testing for *BRCA1* promoter hypermethylation in routine diagnostics may be unnecessary at this time.

It is noteworthy that different populations show different common *BRCA1/2* variants.[43] For example, *BRCA1* genomic deletions are common founder variants in the Dutch population,[44, 45] whereas large deletions in *BRCA2* are rare. CNV-MLPA for *BRCA2* is therefore not routinely performed. In countries in which *BRCA2* exon deletions are more common (eg. Australia and Italy),[45] additional *BRCA2* CNV-MLPA might be necessary.

The wide mutation spectrum seen in *BRCA1/2* and the presence of variants for which the clinical significance is unclear make interpretation of results challenging.[24] Of the six germline *BRCA1/2* variants identified in the COBRA cohort, two were VUSs. Because this category of variants has unclear pathogenicity, it is important that they are discussed in a multidisciplinary team that includes an expert clinical molecular geneticist.[46]

In the training cohort, we showed the importance of optimizing the bioinformatics process for data analysis to prevent variants present in the sequencing data from not being reported automatically. This was also shown by others.[47]

Because *BRCA1/2* screening of ovarian tumor tissue has proved to be a reliable test both in this study and in previous studies,[25, 32] we propose that screening of tumor tissue for *BRCA1/2* variants should be implemented in routine diagnostics as illustrated in Figure 2. Using the tumor screening test to identify women with *BRCA1/2* variants (either germline or somatic in origin) provides an efficient selection method for referral to clinical genetic services. This scheme resembles the previously adopted Lynch syndrome tumor screening program for colorectal and endometrial cancer.[9, 48] When a *BRCA1/2* variant is identified in the tumor screening test, women can be referred for genetic counseling and may subsequently decide whether they want to know if the variant has a germline origin. This scheme is particularly beneficial to those patients (and their relatives) without a *BRCA1/2* variant, as tumor screening will prevent unnecessary distress because of a possible hereditary origin of the EOC. An additional advantage of tumor screening is that subsequent germline analysis only requires verification of a specific variant, avoiding the need (and associated costs) for whole-gene scanning. On the basis of these considerations, implementation of *BRCA1/2* tumor screening in the care pathway of EOC patients may be an efficient and patient-friendly approach.

Although *BRCA1/2* tumor screening proved to be highly sensitive, some technical limitations were observed. Sequencing artifacts present in homopolymer regions prevented the detection of one *BRCA2* variant in the training cohort (Supplemental Figure S3). Previous studies have already highlighted the high rates of error in insertion/deletion calling associated with homopolymer regions.[25, 29, 30, 49, 50] On the basis of data extracted from the Leiden Open Variant Database (http://www.lovd.nl/3.0/home, last accessed October 13, 2017),[28] in combination with our institutional data, we estimate that approximately one homopolymer germline *BRCA1/2* variant in every 250 patients screened could be missed (Supplemental Table S2). Use

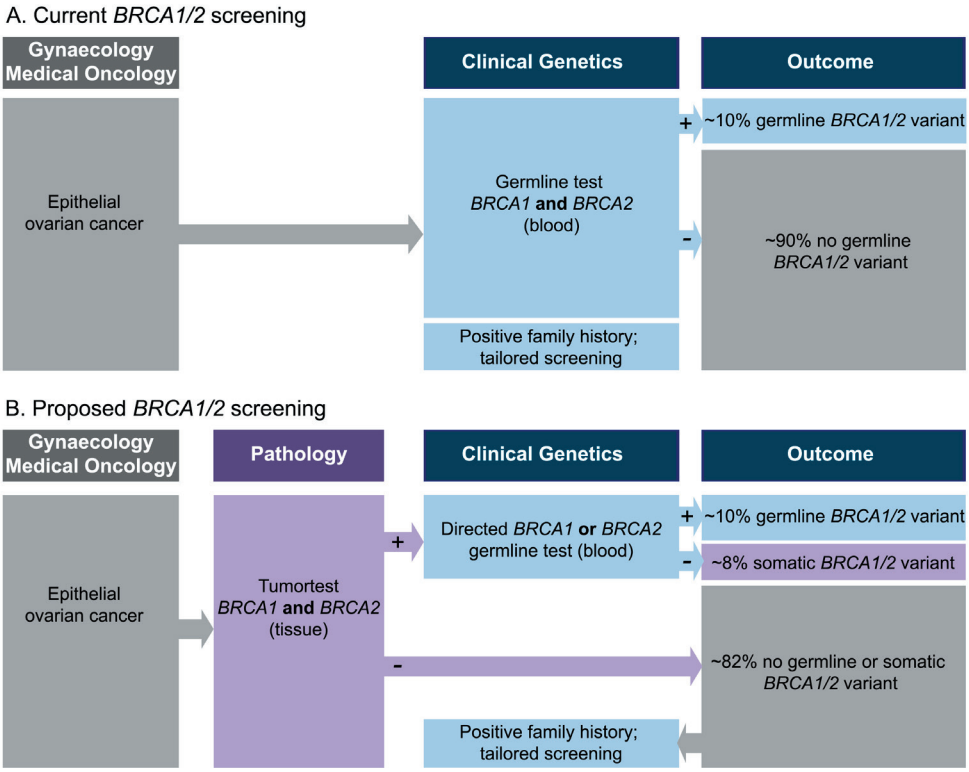


Figure 2. Flowchart illustrating the current epithelial ovarian cancer (EOC) *BRCA1/2* screening pathway (A) and the proposed EOC *BRCA1/2* tumor screening pathway (B). The integration of tumor tissue analysis for *BRCA1/2* variants as part of the ovarian cancer patient pathway is more efficient because it avoids referral of most patients when only those women carrying a *BRCA1/2* mutation or having a suspected family history are being referred for genetic counseling. Percentages are based on the Clinical implementation of *BRCA1/2* screening on ovarian tumor tissue cohort.

of improved sequencing chemistry or sequencing platforms that show better performance with homopolymer regions will mitigate this problem.[50]

A technical limitation, which applies to all amplicon-based sequencing techniques, is the possibility of variants being located at amplicon ends or primer binding sites. Because FFPE-derived DNA is highly fragmented, shorter amplicons are needed, thus increasing the chance of variants being present in amplicon edges or primer locations.

In this study, we optimized and clinically validated a *BRCA1/2* variant tumor screening test of FFPE material. It was demonstrated that the test has adequate sensitivity to detect *BRCA1/2* variants. Therefore, a workflow in which *BRCA1/2* tumor screening is requested by the treating physician and is integrated in routine care for all EOC patients is recommended. This will allow more efficient patient selection for precision medicine, genetic counseling and

preventive options. Awareness of family history remains important and referral to genetic services should be based on both the detection of variants in the tumor test and the presence of affected cases in family histories.

Acknowledgements

We thank Margriet Löwik, Dorien Berends, Margret den Hollander, Carolien Haazer and Clasien Blom for excellent assistance and help with patient inclusion, and Stephanie Schubert for help with figure design.

Supported by AstraZeneca (financial support for next generation sequencing).

Disclosures: None declared.

References

1. Alsop K, Fereday S, Meldrum C, deFazio A, Emmanuel C, George J, et al. BRCA mutation frequency and patterns of treatment response in BRCA mutation-positive women with ovarian cancer: a report from the Australian Ovarian Cancer Study Group. J Clin Oncol 2012;30(21):2654-63.
2. King MC, Marks JH, Mandell JB. Breast and ovarian cancer risks due to inherited mutations in BRCA1 and BRCA2. Science 2003;302(5645):643-6.
3. Norquist BM, Harrell MI, Brady MF, Walsh T, Lee MK, Gulsuner S, et al. Inherited Mutations in Women With Ovarian Carcinoma. JAMA Oncol 2016;2(4):482-90.
4. Cancer Genome Atlas Research Network. Integrated genomic analyses of ovarian carcinoma. Nature 2011;474(7353):609-15.
5. Berchuck A, Heron KA, Carney ME, Lancaster JM, Fraser EG, Vinson VL, et al. Frequency of germline and somatic BRCA1 mutations in ovarian cancer. Clin Cancer Res 1998;4(10):2433-7.
6. Baldwin RL, Nemeth E, Tran H, Shvartsman H, Cass I, Narod S, et al. BRCA1 promoter region hypermethylation in ovarian carcinoma: a population-based study. Cancer Res 2000;60(19):5329-33.
7. Esteller M, Silva JM, Dominguez G, Bonilla F, Matias-Guiu X, Lerma E, et al. Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. J Natl Cancer Inst 2000;92(7):564-9.
8. Martincorena I, Campbell PJ. Somatic mutation in cancer and normal cells. Science 2015;349(6255):1483-9.
9. Lancaster JM, Powell CB, Chen LM, Richardson DL. Society of Gynecologic Oncology statement on risk assessment for inherited gynecologic cancer predispositions. Gynecol oncol 2015;136(1):3-7.
10. Vergote I, Banerjee S, Gerdes AM, van Asperen C, Marth C, Vaz F, et al. Current perspectives on recommendations for BRCA genetic testing in ovarian cancer patients. Eur J Cancer 2016;69:127-34.
11. Lord CJ, Ashworth A. BRCAness revisited. Nat Rev Cancer 2016;16(2):110-20.
12. Roy R, Chun J, Powell SN. BRCA1 and BRCA2: different roles in a common pathway of genome protection. Nat Rev Cancer 2012;12(1):68-78.
13. Marquard AM, Eklund AC, Joshi T, Krzystanek M, Favero F, Wang ZC, et al. Pan-cancer analysis of genomic scar signatures associated with homologous recombination deficiency suggests novel indications for existing cancer drugs. Biomark Res 2015;3:9.
14. Davies H, Glodzik D, Morganella S, Yates LR, Staaf J, Zou X, et al. HRDetect is a predictor of BRCA1 and BRCA2 deficiency based on mutational signatures. Nat Med 2017;23(4):517-525.
15. Naipal KA, Verkaik NS, Ameziane N, van Deurzen CH, Ter Brugge P, Meijers M, et al. Functional ex vivo assay to select homologous recombination-deficient breast tumors for PARP inhibitor treatment. Clin Cancer Res 2014;20(18):4816-26.
16. Konstantinopoulos PA, Ceccaldi R, Shapiro GI, D'Andrea AD. Homologous Recombination Deficiency: Exploiting the Fundamental Vulnerability of Ovarian Cancer. Cancer discov 2015;5(11):1137-54.
17. Kelland L. The resurgence of platinum-based cancer chemotherapy. Nat Rev Cancer 2007;7(8):573-84.
18. O'Sullivan Coyne G, Chen AP, Meehan R, Doroshow JH. PARP Inhibitors in Reproductive System Cancers: Current Use and Developments. Drugs. 2017;77(2):113-30.

19. Ledermann J, Harter P, Gourley C, Friedlander M, Vergote I, Rustin G, 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):852-61.
20. Mirza MR, Monk BJ, Herrstedt J, Oza AM, Mahner S, Redondo A, et al. Niraparib Maintenance Therapy in Platinum-Sensitive, Recurrent Ovarian Cancer. N Engl J Med 2016;375(22):2154-64.
21. Pujade-Lauraine E, Ledermann JA, Selle F, Gebski V, Penson RT, Oza AM, et al. Olaparib tablets as maintenance therapy in patients with platinum-sensitive, relapsed ovarian cancer and a BRCA1/2 mutation (SOLO2/ENGOT-Ov21): a double-blind, randomised, placebo-controlled, phase 3 trial. Lancet Oncol 2017;18(9):1274-1284.
22. Ledermann JA, Harter P, Gourley C, Friedlander M, Vergote I, Rustin G, et al. Overall survival in patients with platinum-sensitive recurrent serous ovarian cancer receiving olaparib maintenance monotherapy: an updated analysis from a randomised, placebo-controlled, double-blind, phase 2 trial. Lancet Oncol 2016;17(11):1579-89.
23. Swisher EM, Lin KK, Oza AM, Scott CL, Giordano H, Sun J, et al. Rucaparib in relapsed, platinum-sensitive high-grade ovarian carcinoma (ARIEL2 Part 1): an international, multicentre, open-label, phase 2 trial. Lancet Oncol 2017;18(1):75-87.
24. Wallace AJ. New challenges for BRCA testing: a view from the diagnostic laboratory. Eur J Hum Genet 2016;24 Suppl 1:S10-8.
25. Endris V, Stenzinger A, Pfarr N, Penzel R, Mobs M, Lenze D, et al. NGS-based BRCA1/2 mutation testing of high-grade serous ovarian cancer tissue: results and conclusions of the first international round robin trial. Virchows Arch 2016;468(6):697-705.
26. Do H, Dobrovic A. Sequence artifacts in DNA from formalin-fixed tissues: causes and strategies for minimization. Clin Chem 2015;61(1):64-71.
27. Shirts BH, Casadei S, Jacobson AL, Lee MK, Gulsuner S, Bennett RL, et al. Improving performance of multigene panels for genomic analysis of cancer predisposition. Genet Med 2016;18(10):974-81.
28. Fokkema IF, Taschner PE, Schaafsma GC, Celli J, Laros JF, den Dunnen JT. LOVD v.2.0: the next generation in gene variant databases. Hum Mutat 2011;32(5):557-63.
29. Shin S, Kim Y, Oh SC, Yu N, Lee ST, Choi JR, et al. Validation and optimization of the Ion Torrent S5 XL sequencer and Oncomine workflow for BRCA1 and BRCA2 genetic testing. Oncotarget 2017;23;8(21):34858-34866.
30. Loman NJ, Misra RV, Dallman TJ, Constantinidou C, Gharbia SE, Wain J, et al. Performance comparison of benchtop high-throughput sequencing platforms. Nat Biotechnol 2012;30(5):434-9.
31. Trujillano D, Weiss ME, Schneider J, Koster J, Papachristos EB, Saviouk V, et al. Next-generation sequencing of the BRCA1 and BRCA2 genes for the genetic diagnostics of hereditary breast and/or ovarian cancer. J Mol Diagn 2015;17(2):162-70.
32. Weren RD, Mensenkamp AR, Simons M, Eijkelenboom A, Sie AS, Ouchene H, et al. Novel BRCA1 and BRCA2 Tumor Test as Basis for Treatment Decisions and Referral for Genetic Counselling of Patients with Ovarian Carcinomas. Hum Mutat 2017;38(2):226-35.

33. Ellison G, Huang S, Carr H, Wallace A, Ahdesmaki M, Bhaskar S, et al. A reliable method for the detection of BRCA1 and BRCA2 mutations in fixed tumour tissue utilising multiplex PCR-based targeted next generation sequencing. BMC Clin Pathol. 2015;15:5.
34. Mafficini A, Simbolo M, Parisi A, Rusev B, Luchini C, Cataldo I, et al. BRCA somatic and germline mutation detection in paraffin embedded ovarian cancers by next-generation sequencing. Oncotarget 2016;7(2):1076-83.
35. van Eijk R, Stevens L, Morreau H, van Wezel T. Assessment of a fully automated high-throughput DNA extraction method from formalin-fixed, paraffin-embedded tissue for KRAS, and BRAF somatic mutation analysis. Exp Mol Pathol 2013;94(1):121-5.
36. Plon SE, Eccles DM, Easton D, Foulkes WD, Genuardi M, Greenblatt MS, et al. Sequence variant classification and reporting: recommendations for improving the interpretation of cancer susceptibility genetic test results. Hum Mutat 2008;29(11):1282-91.
37. Williams C, Ponten F, Moberg C, Soderkvist P, Uhlen M, Ponten J, et al. A high frequency of sequence alterations is due to formalin fixation of archival specimens. Am J Pathol 1999;155(5):1467-71.
38. Sutcliffe S, Pharoah PD, Easton DF, Ponder BA. Ovarian and breast cancer risks to women in families with two or more cases of ovarian cancer. Int J Cancer 2000;87(1):110-7.
39. Davila RE, Rajan E, Baron TH, Adler DG, Egan JV, Faigel DO, et al. ASGE guideline: colorectal cancer screening and surveillance. Gastrointest Endosc 2006;63(4):546-57.
40. Veeck J, Ropero S, Setien F, Gonzalez-Suarez E, Osorio A, Benitez J, et al. BRCA1 CpG island hypermethylation predicts sensitivity to poly(adenosine diphosphate)-ribose polymerase inhibitors. J Clin Oncol 2010;28(29):e563-4; author reply e5-6.
41. Drew Y, Mulligan EA, Vong WT, Thomas HD, Kahn S, Kyle S, et al. Therapeutic potential of poly(ADP-ribose) polymerase inhibitor AG014699 in human cancers with mutated or methylated BRCA1 or BRCA2. J Natl Cancer Inst 2011;103(4):334-46.
42. Lheureux S, Lai Z, Dougherty BA, Runswick S, Hodgson D, Timms KM, et al. Long-term responders on olaparib maintenance in high-grade serous ovarian cancer: Clinical and molecular characterization. Clin Cancer Res 2017;23(15):4086-4094
43. Ferla R, Calo V, Cascio S, Rinaldi G, Badalamenti G, Carreca I, et al. Founder mutations in BRCA1 and BRCA2 genes. Ann Oncol 2007;18 Suppl 6:vi93-8.
44. Petrij-Bosch A, Peelen T, van Vliet M, van Eijk R, Olmer R, Drusedau M, et al. BRCA1 genomic deletions are major founder mutations in Dutch breast cancer patients. Nat Genet 1997;17(3):341-5.
45. Ewald IP, Ribeiro PL, Palmero EI, Cossio SL, Giugliani R, Ashton-Prolla P. Genomic rearrangements in BRCA1 and BRCA2: A literature review. Genet Mol Biol 2009;32(3):437-46.
46. Lindor NM, Goldgar DE, Tavtigian SV, Plon SE, Couch FJ. BRCA1/2 sequence variants of uncertain significance: a primer for providers to assist in discussions and in medical management. Oncologist. 2013;18(5):518-24.
47. Ellison G, Ahdesmaki M, Luke S, Waring PM, Wallace A, Wright R, et al. An evaluation of the challenges to developing tumour BRCA1 and BRCA2 testing methodologies for clinical practice. Hum Mutat 2018;39(3):394-405.

48. Rubenstein JH, Enns R, Heidelbaugh J, Barkun A. American Gastroenterological Association Institute Guideline on the Diagnosis and Management of Lynch Syndrome. BMC Gastroenterol 2015;149(3):777-82.
49. Buzolin AL, Moreira CM, Sacramento PR, Oku AY, Fornari A, Antonio DSM, et al. Development and validation of a variant detection workflow for BRCA1 and BRCA2 genes and its clinical application based on the Ion Torrent technology. Hum Genomics 2017;11(1):14.
50. Goodwin S, McPherson JD, McCombie WR. Coming of age: ten years of next-generation sequencing technologies. Nat Rev Genet 2016;17(6):333-51.

Supplemental tables

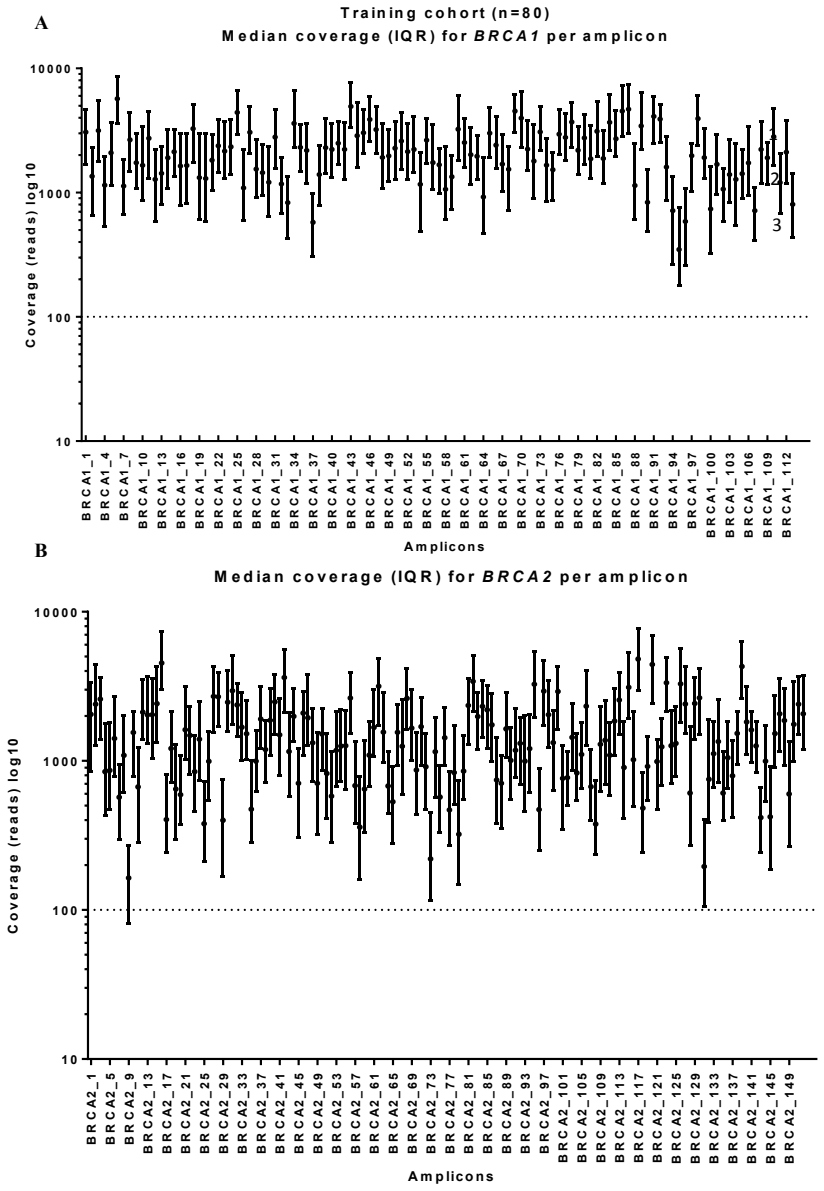
Supplemental Table S1. Samples of insufficient quality for analysis

SampleID	Amplicons covered with 0 reads		Amplicons covered with <100 reads		Reason QC-failed
	Number	%	Number	%	
Training cohort					
R9-tumor	73	27,5	148	55,8	Amplification failure of the majority of amplicons
R16-tumor	18	6,8	237	89,4	Amplification failure of the majority of amplicons and high number of transitions
R16-normal	24	9,1	239	90,2	Amplification failure of the majority of amplicons and high number of transitions
R23-tumor	7	2,6	101	38,1	Amplification failure of the majority of amplicons and high number of transitions
R33-normal	82	30,9	265	100	Amplification failure of the majority of amplicons
R34-tumor	6	2,3	265	100	Amplification failure of the majority of amplicons
R50-tumor	34	12,8	265	100	Amplification failure of the majority of amplicons
COBRA cohort					
p2-normal	36	13,6	129	48,7	Amplification failure of the majority of amplicons
p12-normal	17	6,4	20	7,5	Amplification failure of multiple amplicons
p13-normal	4	1,5	165	62,3	Amplification failure of multiple amplicons
P21-tumor	14	5,3	158	59,6	Amplification failure of the majority of amplicons and high number of transitions
P21-normal	10	3,8	143	54,0	Amplification failure of the majority of amplicons and high number of transitions
P53-normal	118	44,5	265	100	Amplification failure of the majority of amplicons

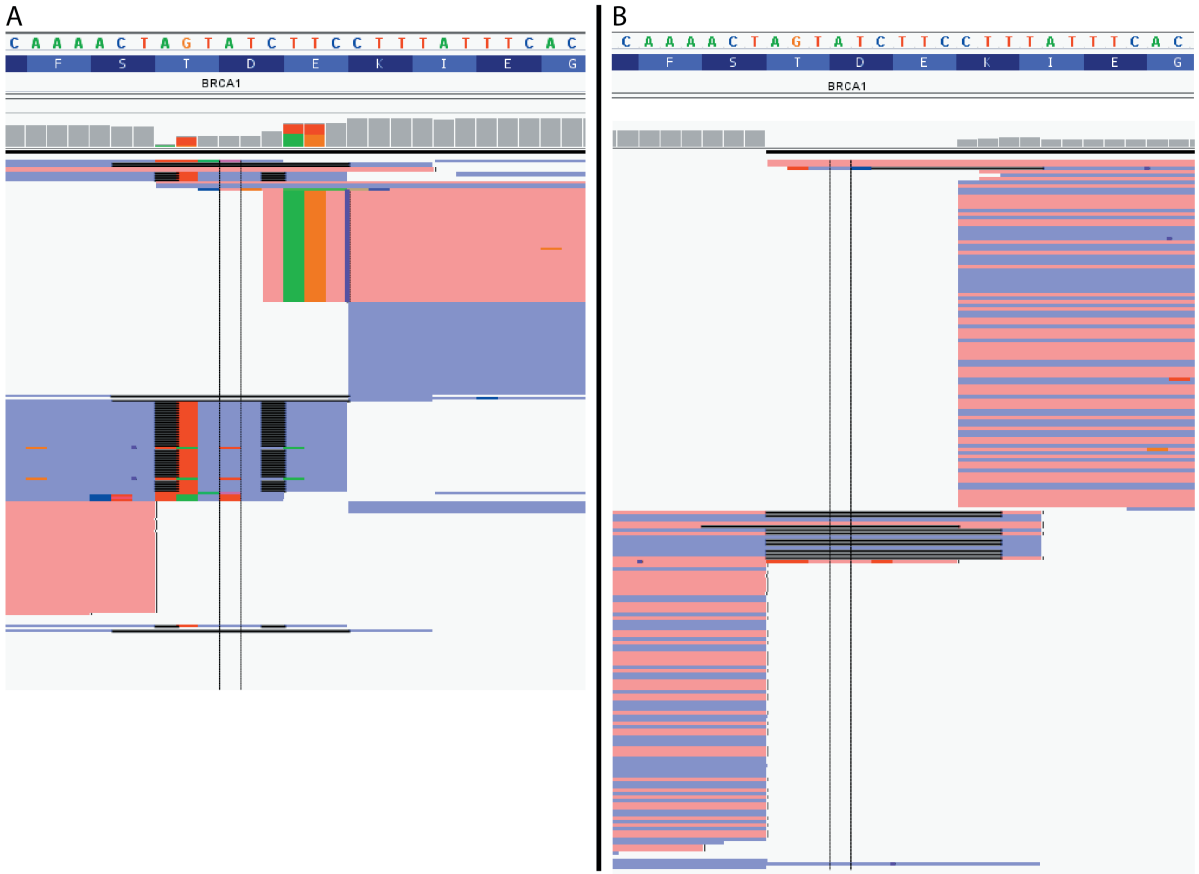
Supplemental Table S2. The 32 homopolymer regions of ≥6 base pairs in the coding sequences of *BRCA1* and *BRCA2*

Genomic Position* *BRCA1*		Stretch	variants †,‡	Frequencies§
41256251	41256256	6A	c.329dup	
41247865	41247870	6A		
41246532	41246538	7A	c.1016del c.1016dup c.1010del	1
41245587	41245594	8A	c.1958_1961del c.1961del c.1961dup	
41244219	41244224	6A	c.3326_3329del c.3329dup	
Genomic position* *BRCA2*		**Stretch**	**Variants†**	**Frequencies‡**
32889779	32889784	6A		
32890628	32890633	6T	c.36del	
32906566	32906571	6A	c.952A>T	
32906603	32906609	7A	c.994del	
32907203	32907208	6A		
32907421	32907428	8A	c.1813dup c.1813del	3 2
32910662	32910667	6A	c.2175dup	
32911074	32911080	7A	c.2588dup	
32911322	32911327	6A	c.2830A>T c.2830del	
32911443	32911449	7A	c.2957del c.2957dup	
32912346	32912352	7A	c.3860del c.3860dup	
32912656	32912661	6T	c.4169del	
32912771	32912776	6T	c.4284dup	1
32913080	32913085	6A		
32913559	32913565	7A	c.5071A>T c.5073del c.5073dup	
32913784	32913789	6A		
32913837	32913843	7A	c.5350_5351del c.5351dup	 1
32914070	32914075	6A		
32914860	32914865	6A		
32929162	32929167	6A		
32937355	32937360	6A		
32953633	32953639	7A		
32954023	32954030	8A	c.9097del c.9097dup	 1
32954273	32954279	7A	c.9253dup	
32972590	32972595	6A		
32972626	32972631	6A		
32972893	32972898	6A		

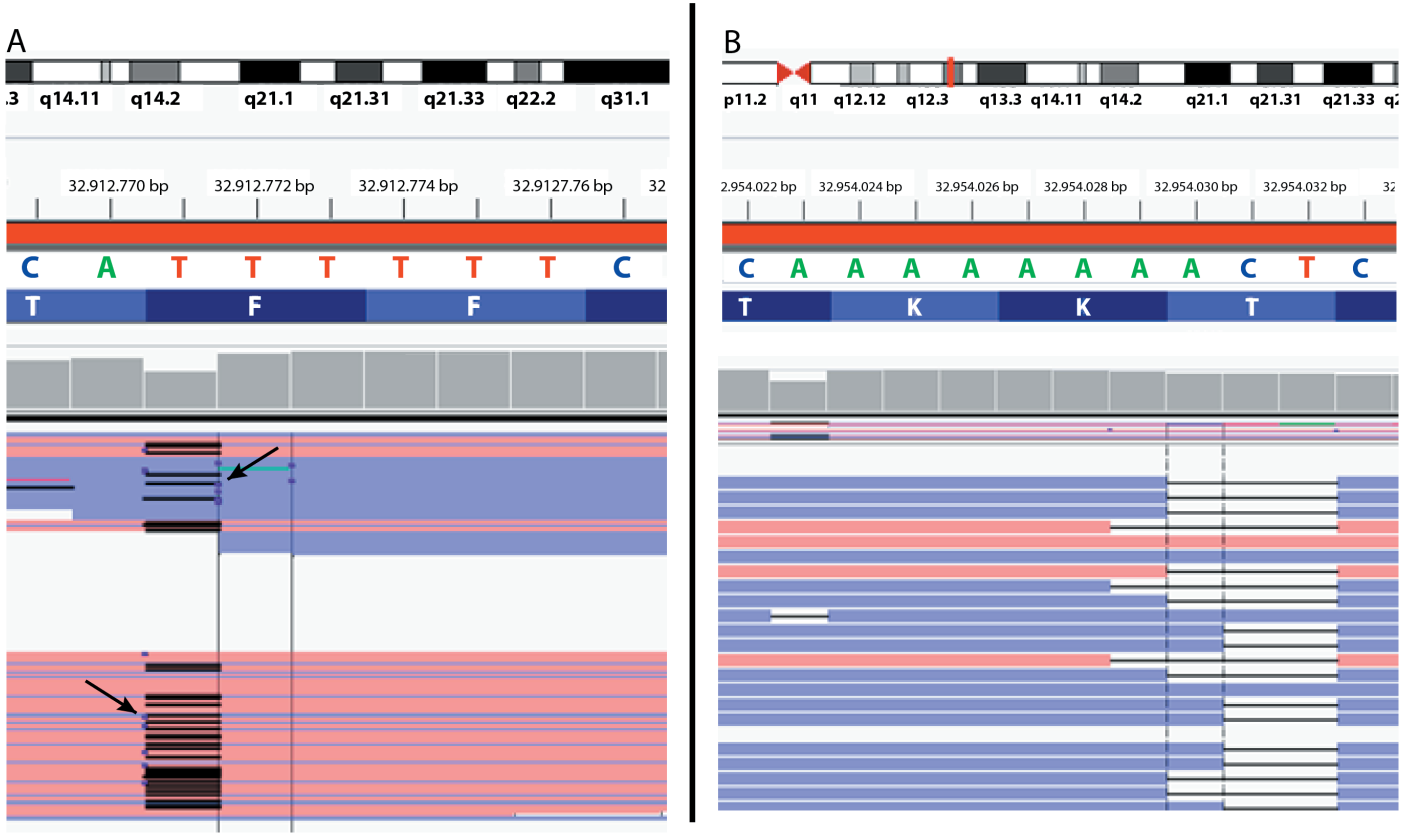
In the LUMC, a class 4 or 5 variant was reported in 236 patients between 2007 and 2016. Of these, nine were variants in homopolymer regions (9/236, 3.8%). Based on the frequency of germline *BRCA1/2* variants observed in the COBRA cohort (9.7%), less than one pathogenic germline variant is estimated to be missed in every 250 patients screened. *Human Reference Consortium 37 (GRCh37). †Reference sequences: *BRCA1:*NM_007294.3, *BRCA2:* NM_ 000059.3 ‡Previously submitted (likely) pathogenic variants extracted from the Leiden Open Variant Database at the genomic positions that are indicated in the table (LOVD; access date: 13-10-2017). §Times detected by LDGA between 2007 and 2016.



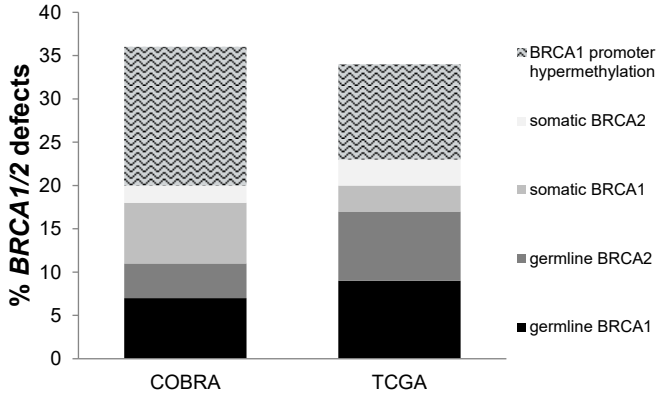
Supplemental Figure S1. Median coverage of the amplicons used to sequence *BRCA1* and *BRCA2* in the training cohort. Error wbars indicate the interquartile range (IQR). The figure only includes the samples that passed quality control. Two samples were excluded from coverage analysis because they were analyzed with an older version of the panel (version 3 instead of version 4). **A:** *BRCA1*; all 113 amplicons have a median coverage >100 reads, with amplicon 95 showing the lowest median coverage, with 348 reads. The median and mean coverage values of all amplicons are 2010 and 2774 reads, respectively. **B:** *BRCA2*; all 152 amplicons have a median coverage >100 reads, with amplicons 9, 73, and 131 showing the lowest median coverage, with 164, 220, and 196 reads, respectively. The median and mean coverage values of all amplicons are 1312 and 1904 reads, respectively. The **dotted line** represents the median coverage >100 reads. *n* = 82 (**A** and **B**, 42 tumor and 40 normal).



Supplemental Figure S2. Adjustments in the alignment improved automatic detection of two variants (R32 and R36). A: Initially, the *BRCA1* deletion (c.3481_3491delGAAGATACTAG) in sample R32 was not automatically detected by the pipeline, and visual inspection of the reads in Integrative Genomics Viewer was needed for identification. **B:** Adjustment of the alignment settings resulted in improved alignment of the reads and automatic detection of the deletion.



Supplemental Figure S3. Sequencing artifacts at the border of homopolymer regions of ≥6 bp interfere with variant detection. A: Sample R24 contains a T duplication (*BRCA2*: NM_000059.3: c.4284dupT) in a stretch of six thymidines. The thymidine duplication is visible in a subset of reads (purple dots; **arrows**) in the Integrative Genomics Viewer but could not be identified because homopolymer instability caused by the T stretch in the form of a bp deletion (black stripes). **B:** Sequencing artifacts present at the border of an 8-bp adenine stretch led the software (Ion Torrent specific caller, Torrent Variant Caller 5.0.2) to call a 2-bp deletion (*BRCA2*: NM_000059.3: c.9099_9100delCT) present in sample R1 as a 3-bp deletion (NM_000059.3: c.9097_9099delACT), which was manually curated.



Supplemental Figure S4. Percentages of *BRCA1/2* defects detected in the high-grade ovarian, fallopian tube, and primary peritoneal cancers (HGSCs) of the clinical implementation of *BRCA1/2* screening on ovarian tumor tissue (COBRA) cohort compared with The Cancer Genome Atlas (TCGA) cohort. Seven percent (4/54) of g*BRCA1* variants, 4% (2/54) of g*BRCA2* variants, 7% (4/54) of s*BRCA1* variants, 2% (1/54) of s*BRCA2* variants, and 16% (8/50) of *BRCA1* promoter hypermethylated cases were detected in HGSCs of COBRA cases included in the final analysis. TCGA found 9% (27/316) of g*BRCA1* variants, 8% (24/316) of g*BRCA2* variants, 3% (10/316) of s*BRCA1* variants, 3% (9/316) of s*BRCA2* variants, and 11% (56/489) of *BRCA1* promoter hypermethylation in HGSCs. Considering the TCGA cohort: One case with both a germline *BRCA1* and *BRCA2* variant was only counted in the germline *BRCA1* mutated group, one case with a simultaneous somatic *BRCA1* variant and germline *BRCA2* variant was only counted in the germline *BRCA2* mutated group, and one case with a simultaneous somatic *BRCA2* variant and germline *BRCA2* variant was only counted in the germline *BRCA2* mutated group.