

Ovarian Cancer-Specific *BRCA*-like Copy-Number Aberration Classifiers Detect Mutations Associated with Homologous Recombination Deficiency in the AGO-TR1 Trial



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ABSTRACT

Purpose: Previously, we developed breast cancer *BRCA1*-like and *BRCA2*-like copy-number profile shrunken centroid classifiers predictive for mutation status and response to therapy, targeting homologous recombination deficiency (HRD). Therefore, we investigated *BRCA1*- and *BRCA2*-like classification in ovarian cancer, aiming to acquire classifiers with similar properties as those in breast cancer.

Experimental Design: We analyzed DNA copy-number profiles of germline *BRCA1*- and *BRCA2*-mutant ovarian cancers and control tumors and observed that existing breast cancer classifiers did not sufficiently predict mutation status. Hence, we trained new shrunken centroid classifiers on this set and validated them in the independent The Cancer Genome Atlas dataset. Subsequently, we assessed *BRCA1/2*-like classification and obtained germline and tumor mutation and methylation status of cancer predisposition

genes, among them several involved in HR repair, of 300 ovarian cancer samples derived from the consecutive cohort trial AGO-TR1 (NCT02222883).

Results: The detection rate of the *BRCA1*-like classifier for *BRCA1* mutations and promoter hypermethylation was 95.6%. The *BRCA2*-like classifier performed less accurately, likely due to a smaller training set. Furthermore, three quarters of the *BRCA1/2*-like tumors could be explained by (epi)genetic alterations in *BRCA1/2*, germline *RAD51C* mutations and alterations in other genes involved in HR. Around half of the non-*BRCA*-mutated ovarian cancer cases displayed a *BRCA*-like phenotype.

Conclusions: The newly trained classifiers detected most *BRCA*-mutated and methylated cancers and all tumors harboring a *RAD51C* germline mutations. Beyond that, we found an additional substantial proportion of ovarian cancers to be *BRCA*-like.

Introduction

Epithelial ovarian cancer is the second leading cause of death among gynecologic cancers worldwide as there were 286,000 incident cases and mortality of 176,000 reported in 2017 (1). Because screening tools are ineffective and early clinical warning signs are rare, the majority of ovarian cancer cases present in late clinical

stages. Even though therapeutic strategies improved within the past years, the prognosis is still poor, with an average 5-year survival rate of 48.6% (2).

Cytoreductive surgery aiming for complete resection, followed by platinum-based chemotherapy, has been the backbone of ovarian cancer treatment for decades (3, 4). Carboplatin, combined with paclitaxel ± bevacizumab, as an initial systemic regimen leads to a

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Translational Relevance

We developed ovarian cancer-specific DNA copy-number aberration classifiers that identify a population enriched for *BRCA1/2* and HR-associated gene mutations and might be promising biomarker candidates for response to HRD-directed treatment.

response rate of approximately 80%, especially in high-grade serous ovarian cancer (HGSOC). Nevertheless, the disease of most patients recurs over time (5).

Recently, PARP inhibitors (PARPi) were added to the therapeutic arsenal. Both the mainstay carboplatin/paclitaxel and these new strategies yield high responses in the overall population, which could be explained by homologous recombination deficiency (HRD) in a substantial proportion of ovarian cancer (6).

Platinum compounds and PARPi exploit the HRD, by inducing DNA double-strand breaks or impeding its repair via synthetic lethality, leading to cell-cycle arrest or death. The breast and ovarian cancer germline predisposition genes *BRCA1* and *BRCA2* play crucial roles in homologous recombination (7, 8), an essential, highly accurate DNA-repair process fixing double-strand breaks. Deleterious germline mutations with subsequent loss of heterozygosity (LOH) in *BRCA1/2* can explain a subset of HR-deficient ovarian cancers, resulting in the registration of *BRCA* mutation analyses as companion diagnostics in specific ovarian cancer (9) and metastasized breast cancer (10) settings. However, it was found that a larger proportion of ovarian cancer displays a phenotype similar to germline (g) *BRCA1/2*-mutated cancers, so-called *BRCAness* (11). This is supported by preclinical analyses (12) and clinical trials of the three registered PARPi in ovarian cancer (13–16) demonstrating survival benefits for a larger subgroup, and has led to their approval, independent of mutation status in most indications. Because these additional patients might benefit from a specific therapy, various potential biomarkers are under investigation, including single gene methylation, gene expression (profiles), copy number/LOH-based assays, mutational signatures, and combinations (17–21).

Of these, the LOH score (ref. 15; Foundation Medicine) and MyChoice (ref. 22; Myriad) were applied within the trials mentioned above and demonstrated the ability to narrow the respective subgroup. This led to the first approval of an HRD assay (MyChoice) as a companion diagnostic for applying niraparib in heavily pretreated patients with ovarian cancer (23). Two recent clinical trials (24, 25) evaluated this biomarker in the first-line setting, showing a benefit of a PARPi predominantly in the HRD-positive, but also in the HRD-negative subgroup. In contrast, within the PAOLA-1 trial (26), the test defined a subpopulation beyond *BRCA* mutation carriers benefitting most from the addition of olaparib to bevacizumab maintenance therapy after carboplatin and paclitaxel. Only recently, the European Medicines Agency recommended the approval of this combination therapy for HR-deficient ovarian cancer defined by the presence of *BRCA* mutations or genomic instability for first-line maintenance treatment accordingly (27). Nonetheless, the exploration of further transparent tests that can easily be implemented and further elaborated on decentral platforms is still ongoing to improve the quest for predictive markers.

In breast cancer, a *BRCA*-like classifier based on tissue-specific copy-number profiles was built (28) to discriminate *BRCA*-associated from sporadic cancers by employing the shrunken centroid algorithm (29). This classifier was successfully validated as a predictive marker for

benefit of high-dose alkylating chemotherapy in stage III HER2/neu-negative breast cancer (30, 31), which could be confirmed in several independent retrospective cohorts (32, 33) and is in validation within a prospective breast cancer trial (34). Its derivative gene expression classifier predicted response to a combination of neoadjuvant veliparib and carboplatin (35) in triple-negative breast cancer.

We apply the same methodologic approach to create ovarian cancer-specific *BRCA1/2*-like classifiers, assuming them to be reliable screening tools for HRD in ovarian cancer. In the current study, we further validate these classifiers within The Cancer Genome Atlas (TCGA) cohort and the independent, well-characterized sample set of the observational AGO-TR1 trial (NCT02222883; refs. 36, 37).

The main goal was to evaluate their predictive power in finding samples with a known HR defect (i.e., *BRCA*-associated tumors). Moreover, we want to identify other genetic and clinical characteristics associated with a *BRCA*-like profile and investigate this phenotype's occurrence within the examined cohorts.

Materials and Methods

Ovarian cancer-*BRCA1/2*-like classifier training

Training cohort

Fifty confirmed *gBRCA1*-mutated (m), 10 confirmed *gBRCA2* m cases, and 13 patients without a family history of ovarian or breast cancer (controls) were identified through the Netherlands Cancer Institute (NKI, Amsterdam, the Netherlands) tumor registration database and the Erasmus Medical Center (see **Table 1**). This study was conducted in concordance with Dutch law and national guidelines that allow for the analysis of residual tissue specimens obtained for diagnostic purposes and anonymized publication of the results (38).

DNA isolation and *BRCA1* methylation assay

Histologic classification and grading, according to the two-tiered system, were performed by CHMvD and PCEG. We isolated DNA from formalin-fixed paraffin-embedded (FFPE) tumor slides, using (micro)dissection to obtain 60% tumor percentage with the Qiagen DNA mini kit (Qiagen, catalog no. 51306) and for *BRCA1* methylation, using an MLPA kit (MRC-Holland) as described before (30), see Supplementary Materials and Methods for extended description.

NimbleGen 135K oligonucleotide array comparative genomic hybridization

We obtained NimbleGen 135K array comparative genomic hybridization (aCGH) profiles from the training set samples. The 135K array data were mapped to the BAC aCGH platform for dimension and noise reduction by averaging probes covered by the BAC clone, as described before (39, 40). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE111688 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE111688>).

Table 1. Pathologic characteristics of samples within the training set.

		Control	<i>gBRCA1</i>	<i>gBRCA2</i>
Subtype	Serous	13	48	10
	Endometrial	0	1	0
	Other	0	1	0
Grade	Low grade	1	1	0
	High grade	12	48	10
	Missing	0	1	0

Estimating the performance of the classifier in a 10-fold double-loop cross-validation loop

We carried out nested double-loop 10-fold cross-validation of shrunken centroid classifiers to classify ovarian cancer as being similar to *gBRCA1* m (*BRCA1*-like) or *gBRCA2* m (*BRCA2*-like) or controls (C; ref. 29). We obtained the delta threshold by optimizing the classification error and selecting the sparsest model within one SE of the optimal solution (41). Subsequently, we used the model at the selected threshold to predict the samples in the outer loop. The AUC of the receiver/operator curve (ROC) of our predictions of the class labels is computed using samples that are left out of the training procedure. After estimating this unbiased performance, we trained the full dataset's final model using the inner loop. See Supplementary Materials and Methods for pseudocode.

Validation in TCGA dataset

We downloaded the Infinium HumanMethylation27 BeadChip methylation, segmented genome-wide human SNP6.0 copy number, and gene expression data from the firebrowse.org archives of the ovarian cancer TCGA data (version 2015082100.0.0). *BRCA1* and *BRCA2* mutation status were obtained from cBioPortal, which stores the somatic and germline mutation status used in the original TCGA articles (42). The *BRCA1* promoter methylation status was obtained by correlating the methylation and gene expression data. We used methylation probe cg10893007 because of the strongest Pearson correlation with log *BRCA1* gene expression ratio among the *BRCA1* promoter probes and close location to the MLPA probe used in the previous study (30) among those covering the *BRCA1* promoter in TCGA dataset.

Classifying TCGA data

TCGA data were mapped to the NKI BAC array CGH positions, as described before (39). In short, SNP6.0 probes within the start and end positions of the BAC clone were averaged (mapped TCGA). We adjusted for differences in scaling and centering by using a method similar to quantile normalization. Briefly, we performed linear regression by fitting a generalized linear Gaussian model with identity link function to the sorted location-wise average DNA copy-number values of the NimbleGen data and the sorted location-wise DNA copy-number averages of the mapped TCGA dataset. Subsequently, we used the obtained alpha coefficient to correct the centering and the obtained beta coefficient to correct the scaling of the mapped TCGA data, followed by classification. We validated this method on samples that had been analyzed before (39) both on NimbleGen 135K aCGH and SNP6.0. See Supplementary Materials and Methods for additional information. Subsequently, samples were classified as *BRCA*-like if the predicted probability was > 0.5 and non-*BRCA*-like if the predicted probability was ≤ 0.5 , as was predefined in the training set.

Performance in TCGA data

The sensitivity and specificity of detecting the class labels *gBRCA1/BRCA2* mutation, *sBRCA1/BRCA2* mutation, and *BRCA1* promoter hypermethylation were calculated. The Youden index, (sensitivity+specificity) – 1, was used as a balanced measure to assess performance. This readout resembles the equal weighting of sensitivity and specificity in the training process.

Independent validation within AGO-TR1 cohort study

Within the consecutive cohort study AGO-TR1 (NCT02222883), 523 patients with ovarian cancer were counseled and enrolled in 20 AGO study group centers in Germany. The ethical committee

approved the study protocol of the Landesärztekammer Nordrhein (Nr. 2014340). Written informed consent was obtained before any study-related procedure. All individuals were 18 years or older and displayed a primary (PR; $n = 281$) or platinum-sensitive relapsed (RE; $n = 242$) ovarian cancer, defined as relapse after a platinum-free interval of at least 6 months. The AGO study group documented clinical data including demographics, medical and family history as well as disease characteristics.

The Center of Hereditary Breast and Ovarian Cancer in Cologne performed the genetic analyses on blood samples of all participants and FFPE tumor samples of 496 patients as described previously (36, 37). In short, a paired multi-gene panel sequencing of germline and tumor samples analyzing 27 ovarian cancer-related and DNA repair genes (see Supplementary Materials and Methods) was performed with a complementary use of copy-number variation analysis for the detection of large genomic rearrangements in germline samples. All variants were classified using a five-tier variant classification system as proposed by the International Agency for Research on Cancer Unclassified Genetic Variants Working Group, namely, deleterious = class 5, likely deleterious = class 4, variant of uncertain significance = class 3, likely benign = class 2 and benign = class 1. For somatic variants, the My Cancer Genome database (<http://www.mycancergenome.org>), the IARC TP53 database (<https://p53.iarc.fr>), and the ClinVar database (<https://www.ncbi.nlm.nih.gov/clinvar/>) were also considered for variant classification. Variants reported to occur in large outbred control reference groups at an allele frequency of $>1\%$ were generally considered benign. Class 4/5 variants were subsequently defined as "deleterious variants." Variants were considered somatic if they were not identified in a paired germline analysis of the corresponding blood sample. Also, quantitative methylation assays analyzing *BRCA1*, *PALB2*, and *RAD51C* promoter regions were carried out as described before (37). In total, the complete data of 473 individuals were successfully generated (ref. 37; see CONSORT-like flow diagram; Supplementary Fig. S1).

Selection of the study sample

We selected 300 samples with matching germline and somatic mutation status and successfully performed methylation analyses for *BRCA*-like classification (see Table 2), including all available samples with deleterious germline and somatic variants (IARC class 4/5) in *BRCA1/2* ($n = 118$), other HR-related and hereditary non-polyposis colorectal cancer (HNPCC) genes ($n = 44$), and somatic (s) *BRCA1/2-VUS* (variants of unknown significance; $n = 4$). As controls, we randomly selected a similarly sized group ($n = 134$) from the rest of the main cohort taking PR/RE status, age at diagnosis, and *sTP53* mutations into account. Samples with only *gBRCA1/2 VUS* ($n = 15$) were excluded.

DNA isolation

Hematoxylin and eosin-stained 3 μm tissue sections were centrally investigated (Institute of Pathology, University Hospital Bonn, Bonn, Germany). Tumor areas containing $>80\%$ tumor nuclei were chosen and dissected for DNA isolation. DNA isolation from FFPE tumor samples was conducted using standard procedures, as described previously (37).

Low-coverage whole-genome sequencing and *BRCA1/2*-like classification

Low-coverage whole-genome sequencing (lcWGS) was centrally performed (NKI, Amsterdam, the Netherlands), as described earlier (39). Library preparation was performed with an input of 200 ng

Table 2. Occurrence of genetic and clinical features within the samples of the presented study cohort^a.

		A		B	
		Study cohort (n)	[%]	Study cohort (n) without BRCA/HR gene mutations	[%]
Germline and somatic Mutation status	<i>gBRCA1</i>	69	23.0		
	<i>gBRCA2</i>	23	7.7		
	gHR genes	36	12.0		
	<i>sBRCA1/2</i>	28	9.3		
	sHR genes	12	4.0		
	gHNPCC genes	3	1.0	3	2.1
	<i>sTP53</i>	251	83.7	106	75.2
	<i>sPTEN/PIK3CA</i>	17	5.7	8	5.7
Methylation status	<i>BRCA1</i> methyl	28	9.3	26	18.4
	<i>RAD51C</i> methyl	4	1.3	2	1.4
Histologic subtype	High-grade serous	246	82.0	110	78.0
	High-grade endometrioid	16	5.3	9	6.4
	Serous, grade unknown	6	2.0	3	2.1
	Low-grade serous	7	2.3	4	2.8
	Low-grade endometrioid	4	1.3	1	0.7
	Clear cell	2	0.7	2	1.4
	Mucinous	4	1.3	3	2.1
	Other/missing	15	5.0	9	6.4
Clinical features	PR	160	53.3	83	58.9
	RE	140	46.7	58	41.1
Total		300		141	

Note: Genetic and epigenetic features coincided within some samples and were listed in each subcategory concerned. The mean age at diagnosis was 57.6 years (range, 18–83 years) in the main cohort and 59.4 years (range, 21–83 years) excluding all *BRCA1/2*-mutated or HR gene-mutated cases.

Abbreviations: PR, primary ovarian cancer; RE, recurrent disease.

^aA lists all samples, whereas B excludes all samples with a *BRCA* or other HR gene mutation, as those were enriched within the study cohort.

double-stranded DNA derived from FFPE-tumor samples using the TruSeqDNA LT Sample Preparation kit (Illumina). Ten explicit indexed samples were equimolarly pooled and sequenced in one lane on an Illumina HiSeq 2500 device (Illumina). Single-read sequencing (read-length 65 bp) was performed with an aimed coverage of 0.5× and sequences were aligned to reference genome GRCh38. Reads were counted in 20 kb non-overlapping bins, corrected for CG bias and corrected for local alignment-bases estimated mappability, resulting in ²log count ratios.

The 20 kb resolution ²log ratios were mapped to the 1 MB resolution input for the classifier (mapped AGO-TR1). This was done by averaging the ²log count ratios within the 1 MB bins (surrounding the BAC clone locations of the BAC platform). Because the classifier's training was performed on oligonucleotide array CGH data, we performed a correction of centering and scaling of the data with the next-generation sequencing (NGS) platform in this study. This correction is akin to quantile normalization and was performed by fitting a linear regression model with Gaussian distribution and identity link function using the R glm function to the sorted location-wise average of the training set and the mapped AGOTR1 dataset. Subsequently, we used the obtained alpha coefficient to correct the centering and the obtained beta coefficient to correct the scaling of the new data (Supplementary Materials and Methods). We validated this method on samples that had been analyzed both on NimbleGen 135K array CGH and NGS in the cross-platform robustness dataset we described previously (39). Subsequently, samples were classified as *BRCA*-like if

the predicted probability was >0.5 and non-*BRCA*-like if the predicted probability was ≤0.5 as was predefined in the training set.

Statistical analyses

Fisher exact and χ^2 test were applied, where appropriate, to calculate the level of significance. All tests were two sided, and a $P < 0.05$ after correction for multiple testing using the Benjamini–Hochberg approach was considered significant.

Results

Classifier training

Description of the cohort

We generated copy-number profiles of 13 control, 50 *gBRCA1* m, and 10 *gBRCA2* m ovarian cancer, and show the pathologic characteristics of these tumors in Table 1. Figure 1 presents the average profiles of *gBRCA1* m and *gBRCA2* m versus control ovarian cancers. Frequent genomic losses and the patterns of copy-number aberration in our dataset are similar to other datasets, for example, the shape and occurrence of the gain on chromosome 8q, the loss on 4q, and the loss of chromosome X (43, 44).

Training ovarian cancer-specific classifiers

First, we classified the ovarian cancer copy-number profiles with the breast cancer *BRCA1*-like and *BRCA2*-like classifiers (30, 31) and obtained an AUC of 0.51 [95% confidence interval (CI): 0.4–0.63] for

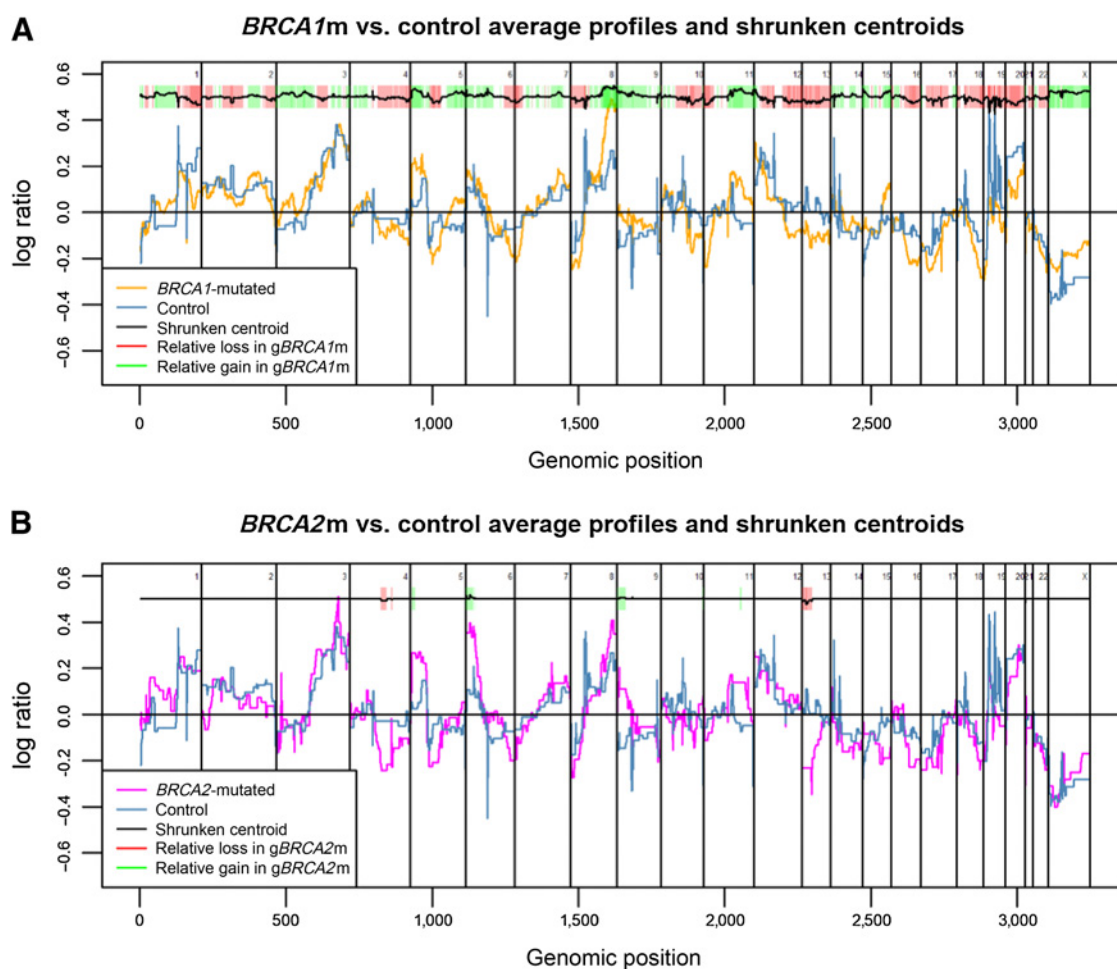


Figure 1.

Average copy-number aberration profiles of *BRCA1*- and *BRCA2*-mutated ovarian cancer. **A**, Average copy-number aberration profile of 50 *gBRCA1* m ovarian cancers and 13 control ovarian cancers. On the *x*-axis, the cumulative genomic position, in megabases, and the *y*-axis, the average $^2\log$ ratio of tumor over normal DNA. **B**, Average copy-number aberration profile of 10 *gBRCA2* m ovarian cancers and 13 control ovarian cancers. On the *x*-axis, the genomic position and on the *y*-axis the average $^2\log$ ratio of tumor DNA over normal DNA.

gBRCA1 m and an AUC of 0.63 (95% CI: 0.28–0.98) for *gBRCA2* m ovarian cancer in the training set. Given this low performance, we trained shrunken centroid classifiers on ovarian cancer data to investigate whether the performance could be improved. These shrunken centroids select genomic regions and weights that are discriminative for the *BRCA*-like and non-*BRCA*-like class. We used ten-fold cross-validation and trained on the class labels *gBRCA1* m versus control and *gBRCA2* m versus control. We observed a cross-validated AUC of 0.67 (0.55–0.78) and 0.91 (0.79–1), respectively, for *BRCA1*-like and *BRCA2*-like classification (Table 3).

Because the NimbleGen aCGH platform is not available anymore, we validated cross-platform compatibility of our classifier using the same methods and dataset as described previously (39); see Supplementary Materials and Methods.

External validation of ovarian cancer BRCA classifiers in TCGA data

We used TCGA ovarian cancer data as an external validation set for our newly trained ovarian cancer classifiers. We classified 358 of 583 samples as having a *BRCA1*-like profile. Within these 358, 25/

26 *gBRCA1*, 10/11 somatic *BRCA1*-mutated tumors, and 67/69 *BRCA1*-methylated tumors were classified as *BRCA1*-like, resulting in an overall sensitivity of 96.2% (95% CI: 0.90–0.99). Specificity, however, is lower, at 40% (95% CI: 0.36–0.45). 350 of 583 samples were assigned to be *BRCA2*-like. Within these 350, 20/25 *gBRCA2* m and 8/11 *sBRCA2* m samples were classified as *BRCA2*-like, resulting in a sensitivity of 77% (95% CI: 0.6–0.9) and a specificity of 41% (95% CI: 0.37–0.49).

Validation within the AGO-TR1 cohort

We further analyzed both the germline/somatic mutation status of *BRCA1/2* and *BRCA1* promoter hypermethylation in the AGO TR1 study. In addition, we aimed to characterize those patients called *BRCA*-like without a mutation in *BRCA1* or *BRCA2*.

Study sample

In total, 300 samples with complete genetic and epigenetic information available, were analyzed. A total of 130 germline and 43 somatic deleterious variants in *BRCA1/2* and other HR genes were present in 159 ovarian cancer samples (see Table 2). The majority

Table 3. Cross-validated results of training shrunken centroids classifiers to distinguish *BRCA1*- or *BRCA2*-mutated from control ovarian cancers.

	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)		Control	<i>BRCA1</i> m	<i>BRCA2</i> m
<i>gBRCA1</i>	0.67	0.76	0.69	Predicted control	9	12	0
vs. control	(0.55–0.78)	(0.62–0.87)	(0.39–0.91)	Predicted <i>BRCA1</i>	4	38	0
<i>gBRCA2</i>	0.91	0.90	0.92	Predicted control	12	0	1
vs. control	(0.79–1)	(0.55–1)	(0.64–1)	Predicted <i>BRCA2</i>	1	0	9

Abbreviations: AUC, area under the curve of the receiver operating characteristic; CI, confidence interval.

displayed a *BRCA1* mutation (g: $n = 69$, s: $n = 17$), a *BRCA2* mutation (g: $n = 23$, s: $n = 11$), or a *RAD51C* mutation (g: $n = 13$). One of these samples presented a germline double mutation of *gBRCA1* and *gBRCA2* and was analyzed with the *gBRCA1* m group; one somatic *BRCA2* mutation coincided with a *gBRCA2* mutation and was assigned to the *gBRCA2* group. Seven other samples with a *BRCA* mutation (g: $n = 5$, s: $n = 2$) displayed a class 4/5 in another HR gene as well and were analyzed with the respective *BRCA*-mutated group.

Most ovarian cancers presented with high-grade serous histology (82%), and a tumor *TP53* mutation was found in 83.7%. Promoter hypermethylation was detected in 9.3% of samples for *BRCA1* ($n = 28$) and in 1.3% of samples ($n = 4$) for *RAD51C*. The mean age at diagnosis was 57.6 years, and 160 primary ovarian cancer and 140 platinum-sensitive recurrent cases were included.

A total of 223 tumors showed a *BRCA1*-like or *BRCA2*-like classification (74.3%). Seventy-seven ovarian cancer thereof were only *BRCA1*-like, 26 were only *BRCA2*-like, and 120 were both *BRCA1*- and *BRCA2*-like.

Detection of *BRCA*-associated ovarian cancers with *BRCA*-like classification

As there is no established gold standard to define *BRCA*ness in general, we first focused on the detection rate of tumors derived from deleterious germline mutations in *BRCA1/2* (see **Table 4**; **Fig. 2**). 67 of 69 *BRCA1*-mutated cases had a *BRCA1*-like (detection rate of 97.1%), whereas 16 of 22 samples with a *BRCA2* class4/5 variant in germline were *BRCA2*-like (detection rate of 72.7%). All remaining *BRCA2*-associated cases were identified by combining both classifiers, that is, being *BRCA1*- and/or *BRCA2*-like.

Regarding deleterious somatic variants, 15 of 17 (88.2%) samples with a *BRCA1* mutation were identified by the *BRCA1*-like classifier. As the other two tumors displayed a *BRCA2*-like phenotype, both classifiers' application detected 100% of the mutated cases. The *BRCA2*-like classifier confirmed 6 of 10 *sBRCA2* m cases. The *BRCA1*-like classifier found no additional sample.

Of the 28 examined samples with a *BRCA1* promoter hypermethylation, 27 displayed a *BRCA1*-like profile (96.4%), one was classified as non-*BRCA*-like.

To better understand the reason for the non-*BRCA*-like classification of the samples mentioned above, we took a closer look at their molecular genetic background (see Supplementary Table S1).

Within the *gBRCA1* m cohort, the non-*BRCA*-like tumors harbored LOF (loss of function) mutations. Both tumors did not show a relevant elevation of variant allele frequency (VAF) of the deleterious *BRCA1* variant in the tumor than germline and none or only a low frequent *sTP53* mutation. One of these *BRCA1* variants was found in two other samples, which were classified as *BRCA1*- and *BRCA2*-like.

Regarding the *sBRCA2* m samples, two of the four non-*BRCA*-like cases displayed several deleterious class 4/5 variants in other analyzed genes, next to the *sBRCA2* mutation with a VAF of 10%, respectively, 12%, but no *sTP53* mutation. Within the *BRCA1*-methylated tumor, no further deleterious somatic alteration was found.

Classification of samples with HR gene alterations beyond *BRCA1/2* and mutations in HNPCC genes

Alterations in further HR genes (*ATM*, *BARD1*, *BRIP1*, *CHEK1*, *CHEK2*, *FAM175A*, *FANCM*, *MRE11A*, *NBN*, *PALB2*, *RAD50*, *RAD51C*, *RAD51D*, *XRCC2*) varied in being classified as *BRCA*-like (see Supplementary Table S2).

Table 4. Detection rate of the *BRCA1*- (A) and *BRCA2*-like classifier (B) in *BRCA*-associated cancers. (C) shows the complementary application of both classifiers; *BRCA1*- and/or *BRCA2*-like.

A)			
	<i>BRCA1</i> -like	[%]	Total
<i>gBRCA1</i>	67	97.1	69
<i>sBRCA1</i>	15	88.2	17
<i>BRCA1</i> methyl	27	96.4	28
<i>BRCA1</i> WT	88	47.3	186
Total	197	65.7	300
B)			
	<i>BRCA2</i> -like	[%]	Total
<i>gBRCA2</i>	16	72.7	22
<i>sBRCA2</i>	6	60.0	10
<i>BRCA2</i> WT	124	46.3	268
Total	146	48.7	300
C)			
	<i>BRCA1/2</i> -like	[%]	Total
<i>gBRCA1</i>	67	97.1	69
<i>gBRCA2</i>	22	100.0	22
<i>sBRCA1</i>	17	100.0	17
<i>sBRCA2</i>	6 ^a	60.0	10 ^a
<i>BRCA1</i> methyl	27 ^a	96.4	28 ^a
<i>BRCA</i> WT	85	54.8	155
Total	223	74.3	300

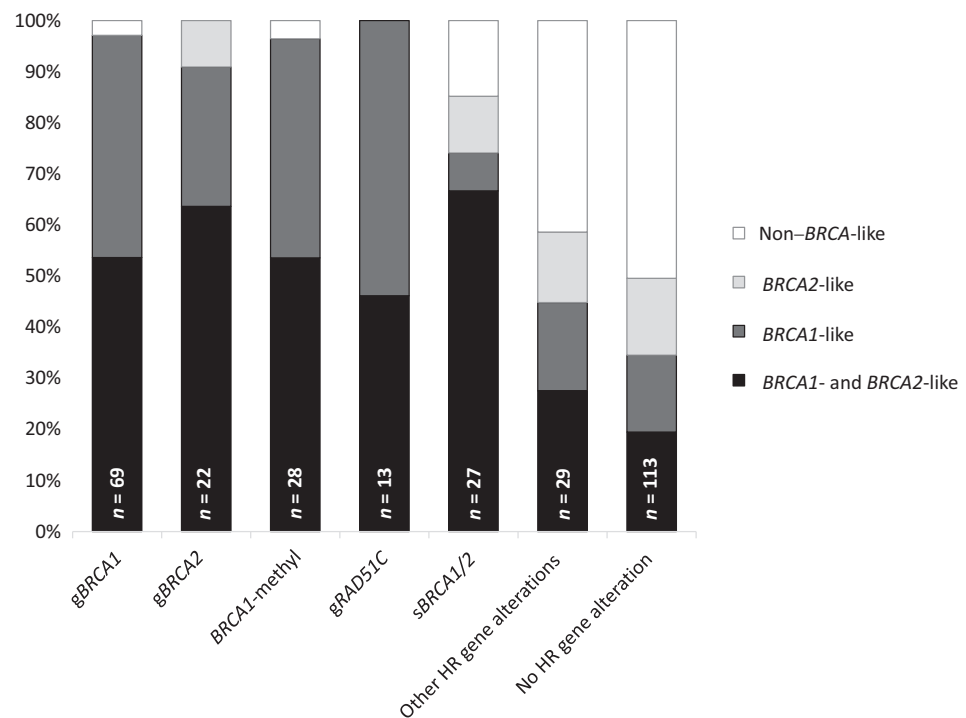
Abbreviations: g, germline; s, somatic; methyl, promoter hypermethylation; WT, wild type.

^aOne sample displayed both a somatic *BRCA2* mutation and a *BRCA1* methylation and was classified as *BRCA1*- and *BRCA2*-like. Therefore, it was presented in both groups but counted only once for the total sum.

The sample displaying both a *gBRCA1* and 2 mutation was counted among the *gBRCA1* samples; the sample with a *BRCA2* mutation in germline and tumor was counted among the *gBRCA2* samples.

Figure 2.

BRCA-like phenotype according to molecular genetic background. Each bar shows the distribution by percentage (y-axis) of BRCA1- and BRCA2-like, only BRCA1-like, only BRCA2-like, and non-BRCA-like profiles within the presented molecular genetic subgroup (x-axis). methyl, promoter hypermethylation; WT, wild type.



Within the study cohort, 44 tumors without *BRCA* mutation or *BRCA1* methylation displayed a genetic alteration in another ovarian cancer risk- or HR gene (except *sTP53* mutations), of which 30 were classified as *BRCA*-like (68.2%; only *BRCA1*-like $n = 11$, only *BRCA2*-like $n = 4$, *BRCA1*- and *BRCA2*-like $n = 15$).

A distinct statistical association was observed for the largest subgroup, the *RAD51C*-associated tumors. All germline mutation-carriers developed a *BRCA*-like ovarian cancer (only *BRCA1*-like $n = 6$, *BRCA1*- and *BRCA2*-like $n = 7$).

No clear statistical association could be observed for most other HR gene mutated samples and samples with *RAD51C* methylation (see Supplementary Table S2; Fig. 2) possibly due to small numbers and coinciding aberrations.

Samples with a class 4/5 variant in *RAD51D* (g: $n = 3$, s: $n = 1$) presented a *BRCA1*- and *BRCA2*-like profile in three of four cases and *FANCM*-associated ovarian cancer (g: $n = 2$, s: $n = 3$) in three of five samples, one sample further presented with only a *BRCA1*-like phenotype. All four *ATM*-associated samples showed a *BRCA*-like profile.

Three of six *PALB2*-associated cancers presented a *BRCA*-like profile, including two samples with each an *ATM* mutation (see above) and a *BRCA1* methylation. Therefore, the underlying mechanism leading to a *BRCA*-like phenotype was unclear.

Samples with each a mutation in *CHEK1* (g), *FAM175A* (g), and *BARD1* (s) as well as three tumors with a germline mutation in HNPCC genes (*MSH2* $n = 2$, *MSH6* with additional somatic *MSH6*- and *BRIP1* mutation, $n = 1$) were examined and showed no *BRCA*-like profile.

Epigenetic alterations in *RAD51C* were rare. Four samples with a *RAD51C* promoter hypermethylation were analyzed, of which one was derived from a germline *BRCA1* mutation carrier, presenting a *BRCA1*- and *BRCA2*-like profile, assumingly due to the known mutation. Of the three remaining samples, one was classified as *BRCA1*- and *BRCA2*-like; the other two (one of them from a *GMRE11A* mutation carrier) displayed a non-*BRCA*-like phenotype.

Molecular genetic background of BRCA-like samples

Within the cohort, 223 samples displayed a *BRCA1/2*-like phenotype. These profiles were associated with a germline mutation in *BRCA1/2* in 89 and a somatic mutation in *BRCA1/2*, respectively a *BRCA1* promoter hypermethylation in 49 of these samples. In another 29 different samples, a somatic or germline class 4/5 variant in another HR gene (esp. *RAD51C*) or a *RAD51C* promoter hypermethylation can explain the presence of a *BRCA*-like phenotype.

For the remaining 55 *BRCA1/2*-like samples (24.6%), no aberration affecting the HR could be detected by gene panel or methylation analyses (see Fig. 3). Regarding all ovarian cancer without a genetic or epigenetic alteration in *BRCA1/2* ($n = 155$), 54.8% ($n = 85$) display a *BRCA1/2*-like phenotype. Excluding all HR gene-mutated cases ($n = 113$), as the study cohort was enriched for those, the *BRCA1/2*-like rate was 48.7% ($n = 55$).

Furthermore, a *BRCA*-like phenotype was correlated with high-grade serous histology, *sTP53* mutations and could be observed tendentially more often, when the patient was included with platinum-sensitive recurring disease than at primary diagnosis. A non-*BRCA*-like profile was seen in association with low-grade serous histology, in combination with *PTEN* or *PIK3CA* mutations and with gHNPCC mutations (see Table 5).

Discussion

In this study, we identified *BRCA1*-like and *BRCA2*-like copy-number classifiers in analogy to those we previously trained in breast cancer (30, 31). Furthermore, we showed in an independent cohort that these signatures identify patients with germline and somatic mutations and promoter hypermethylation in *BRCA1/2*. In addition, we were able to investigate underlying molecular mechanisms in *BRCA*-like cases without a deleterious variant in *BRCA1* or *BRCA2*.

Our approach is supported by the analogy to breast cancer *BRCA*-like classifiers. We previously showed that these assays predicted both

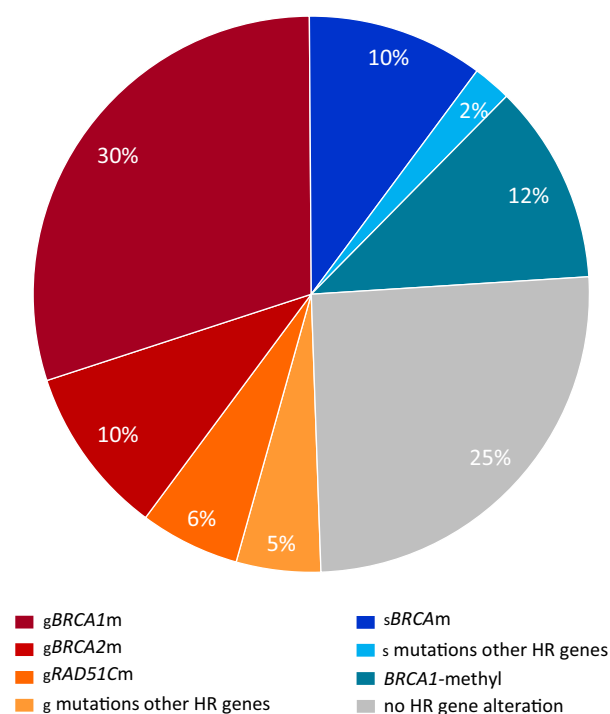


Figure 3.

Molecular genetic background of *BRCA*-like cases. A total of 223 samples are classified as *BRCA1*- and/or *BRCA2*-like. The pie chart shows the portion of samples with a specified (epi-)genetic alteration of a gene, involved in homologous recombination in this subgroup. In 25% of the cases, no HR-affecting variant was detected by NGS or methylation analyses. methyl, promoter hypermethylation.

mutation status and sensitivity to DNA-damaging agents (28, 30, 31). We hypothesized that in ovarian cancer, with the same genetic driver, a similar signature would arise. Applying the breast cancer classifiers in ovarian cancer, we observed that those did not predict mutation status sufficiently. Therefore, we set out to train ovarian cancer-specific classifiers that we validated as being robust with an array- and sequencing-based input data.

Another advantage of this study is that after initial promising results within cross-validation of the training set and in TCGA test dataset were obtained, the AGO-TR1 study provided a large and well-characterized cohort in which we could validate the prediction of mutation status.

Overall, the *BRCA1*-like classifier showed a convincing performance with detecting *BRCA1*-mutated and *BRCA1*-methylated cancers in more than 90% of the cases. When applying both classifiers in the AGO-TR1 trial, more than 95% of all *BRCA1/2*-associated cancers in the cohort were detected. Extensive germline and tumor genetic analysis provided additional support for the *BRCA*-like class showing alterations in *BRCA1/2* or other HR genes in 75% of cases.

Germline *RAD51C* mutations demonstrated a clear association with a *BRCA1*-like profile. For most other HR gene mutations, no such correlation could be proven, possibly due to low sample size or because hereditary predisposition does not always lead to a tumor with HRD (45), stressing the need for complementary germline/tumor analyses to address therapeutic relevance as well as hereditary predisposition.

We detected a *BRCA*-like phenotype in three-quarters of the AGO-TR1 ovarian cancer samples. This is more than in the recently published randomized controlled trials PAOLA-1 (26) and PRIMA (25), where the rate was about half, and might lead to suspicion of overcalling in the current study. However, the current substudy applied skewed selection criteria, so most cases with a *BRCA1/2* mutation (118/126; 93.7%) or a mutation in one of the other HR genes (41/42; 97.6%) were analyzed by lcWGS and only around 50% of the cases without *BRCA*- or HR-related mutation. If we would extrapolate the findings in the selected population to the full AGO TR1 trial, the proportions are entirely in line with other trials (14, 24–26). Regarding the percentage of primary HGSOc responding to platinum compounds of 80% or above, the HR-deficient population in ovarian cancer might even be within those ranges. Yet, this remains to be investigated in a study for predictive value.

Another point of attention is the possible failure to detect *BRCA1*-associated tumors despite the detection rate of over 90%. One reason for this might be the contamination of normal tissue within the sample, which might have been the case with two non-*BRCA*-like *sBRCA2*-mutated tumors and the two *gBRCA1*-mutated tumors (see Supplementary Table S1), which also displayed a noisy profile (see Supplementary Materials and Methods). However, displaying a non-*BRCA*-like profile when carrying a deleterious *BRCA* mutation could also be the correct interpretation, for example, if no second hit/LOH is observed, in which the patient might have developed a sporadic, HR-proficient tumor in the presence of a germline mutation (45).

The *BRCA2*-like classifier was less predictive of *BRCA*/HR gene mutation status, which might be caused by the smaller training set of *gBRCA2*-mutated samples. As this classifier alone is insufficient to predict *BRCA2* mutation status, training a classifier with a more extensive set of *gBRCA2*-mutated samples might be required. In its current state, it only serves to detect samples with a *gBRCA2* mutation and *sBRCA1/2* mutations missed by the *BRCA1*-like classifier.

In the literature, several other assays have been described measuring HRD in ovarian cancer (15, 17, 21, 46), some of which are also partly based on derivatives of SNP/copy-number profiles. Our approach differs from other tests because it uses genomic location-specific aberrations. This could lead to additional information being retained, such as aberrations that might collaborate with the underlying HRD mechanism. In addition, the presented classifiers are tissue specific, which might be an advantage compared with other HRD assays, as a response to targeted strategies can depend on the tissue of origin. An example of this phenomenon is the effect of *BRAF*(V600E) inhibition of *BRAF*(V600E)-mutant cancer in melanoma versus colon cancer (47). Copy-number profiles are generally considered static, low-resolution snapshots. Mutational signatures derived from deeper more in-depth sequencing techniques might identify more directly the traces of errors fixed in the absence of HR and lead to more specific profiles than copy-number profiling (18, 48). While signatures better describe the history of HRD (49), tests like the *RAD51* foci formation assay might yield direct information on the functionality of the HR pathway (50). Furthermore, an analysis in the therapeutic setting, preferably direct comparison of different assays, is mandatory to identify those with the best discriminatory power to predict HRD-specific therapy. For clinical application, availability, adaptability, and cost-effectiveness should be taken into account.

A limitation of the current study is that the gold standard for true HR deficiency should be determined with therapy response, which was unavailable. Although the recognition of mutations in *BRCA1/2* and

Table 5. Rate of BRCA1-, BRCA2-like status, and either according to genetic (germline and somatic), epigenetic, and clinical features^a.

	<i>n</i>	BRCA1-like	Non-BRCA1-like	BRCA1-like [%]	<i>P</i>	BRCA2-like	Non-BRCA2-like	BRCA2-like [%]	<i>P</i>	BRCA1/BRCA2-like	Non-BRCA1/BRCA2-like	BRCA1/BRCA2-like [%]	<i>P</i>
All	300	197	103	65.7	34.3	146	154	48.7	51.3	223	77	74.3	25.7
<i>gBRCA1</i>	69	67	2	97.1	2.9	37	32	53.6	<0.001	67	2	97.1	<0.001
<i>gBRCA2</i>	22	20	2	90.9	9.1	16	6	72.7	0.023	22	0	100.0	0.009
<i>gHRgenes</i>	31	21	10	67.7	32.3	14	17	45.2	1.000	24	7	77.4	0.125
<i>gRAD51C</i>	13	13	0	100.0	0.0	7	6	53.8	0.015	13	0	100.0	0.050
<i>sBRCA1</i>	17	15	2	88.2	11.8	15	2	88.2	0.114	17	0	100.0	0.021
<i>sBRCA2</i>	10	5	5	50.0	50.0	6	4	60.0	0.457	6	4	60.0	0.341
<i>sHRgenes</i>	10	5	5	50.0	50.0	4	6	40.0	0.457	6	4	60.0	0.341
<i>BRCA1-meth</i>	28	27	1	96.4	3.6	15	13	53.6	<0.001	27	1	96.4	0.010
<i>RAD51C-meth</i>	3	1	2	33.3	66.7	1	2	33.3	0.450	1	2	33.3	0.246
no HR gene alteration	113	38	75	33.6	66.4	39	74	34.5	<0.001 ^b	55	58	48.7	<0.001 ^b
<i>gHNPCCgenes</i>	3	0	3	0.0	100.0	0	3	0.0	0.079 ^b	0	3	0.0	0.036 ^b
<i>sTP53</i>	251	185	66	73.7	26.3	138	113	55.0	<0.001	206	45	82.1	<0.001
<i>sPIK3CA</i>	11	3	8	27.3	72.7	1	10	9.1	0.003 ^b	4	7	36.4	0.021 ^b
<i>sPTEN</i>	10	2	8	20.0	80.0	4	6	40.0	0.079 ^b	5	5	50.0	0.217
<i>sPIK3CA/PTEN</i>	17	4	13	23.5	76.5	4	13	23.5	0.001 ^b	7	10	41.2	0.010 ^b
HGS	246	172	74	69.9	30.1	128	118	52.0	0.011	191	55	77.6	0.036
HGE	16	6	10	37.5	62.5	8	8	50.0	0.072 ^b	9	7	56.3	0.272
LGS	7	1	6	14.3	85.7	0	7	0.0	0.030 ^b	1	6	14.3	0.011 ^b
LGE	4	1	3	25.0	75.0	3	1	75.0	0.192	3	1	75.0	0.100
CC	2	0	2	0.0	100.0	0	2	0.0	0.192	0	2	0.0	0.173
muc	4	3	1	75.0	25.0	1	3	25.0	1.000	3	1	75.0	1.000
serous, G unknown	6	3	3	50.0	50.0	2	4	33.3	0.560	4	2	67.7	1.000
other/missing	15	11	4	73.3	26.7	4	11	26.7	0.674	12	3	80.0	1.000
PR	160	97	63	60.6	39.4	75	85	46.9	0.052 ^b	113	47	70.6	0.145
RE	140	100	40	71.4	28.6	71	69	50.7	0.052	110	30	78.6	0.145

Note: In the following somatic *TP53*, *PIK3CA*, and *PTEN* mutations, the histologic subtype and the presence of primary or recurrent disease were analyzed irrespective of alterations in the HR-pathway. As *sTP53*, *sPIK3CA*, and *sPTEN* mutations could co-occur, the affected samples were mentioned in each subcategory. *P* < 0.05 is considered significant (Benjamini-Hochberg correction was applied). Abbreviations: CC, clear cell cancer; G, grading; g, germline; LGE, low-grade endometrioid OC; LGS, low-grade serous OC; HGE, high-grade endometrioid OC; HGS, high-grade serous ovarian cancer (OC); HNPCC, hereditary non-polyposis colorectal cancer; HR, homologous recombination; methyl, promoter hypermethylation; muc, mucinous OC; PR, primary OC; RE, platinum-sensitive recurrent OC; s, somatic.

^aIn the first section, alterations in the HR-pathway are in focus. The sample with both a germline mutation in *BRCA1* and *BRCA2* is analyzed within the *gBRCA1* group, the sample with the germline and somatic deleterious variant in *BRCA2* with the *gBRCA2* group, and the samples with an additional HR gene mutation (*n* = 7) or a *RAD51C* methylation (*n* = 1) in the presence of a *g/sBRCA* mutation are assigned to the respective *BRCA* group as the latter is supposed to be the superordinate feature in developing a *BRCA*-like profile. Three other samples with two mutual alterations (*BRCA1*-methyl and *sBRCA2*, *BRCA1*-methyl and *sPALB2*, *RAD51C*-methyl and *gMRE11A*) were mentioned in both categories, as the predominant mechanism was not obvious. Two of the three gHNPCC samples are included in the "no HR gene alteration" group as well, the other one within the "sHR gene" group as it harbored an *sBRIP1* mutation additionally.

^b*P* values with a significant correlation with non-*BRCA*-like status.

other HR genes that might explain the *BRCA*-like signature is promising, follow-up studies should be done to determine predictive value.

In conclusion, we have shown a convincing performance of the *BRCA1*-like classifier and the combination of both classifiers in detecting *BRCA* mutations/methylation. In addition, around half of the non-*BRCA*-associated ovarian cancer cases displayed a *BRCA*-like phenotype as well. Therefore, it would be interesting to investigate, if the classifiers could also serve as predictive markers of HRD-directed therapy like PARPi, which has to be evaluated in subsequent clinical studies.

Authors' Disclosures

P.C. Schouten reports a patent for *BRCA*-like classifier in ovarian cancer pending and partner is employed by AstraZeneca. L. Richters reports grants from AGO Research GmbH during the conduct of the study; in addition, L. Richters has a patent for *BRCA1/2*-like classifier pending and was supported by the program for Temporary Substitutes for Clinicians (Gerok substitute positions) at the Faculty of Medicine, University of Cologne. C. Ernst reports grants from AGO Research GmbH during the conduct of the study; in addition, C. Ernst has a patent for *BRCA1/2* classifier pending. F. Marmé reports other support from AGO Research GmbH during the conduct of the study as well as personal fees from Roche, AstraZeneca, GSK/Tesaro, PharmaMar, Amgen, GenomicHealth, CureVac, EISAI, Clovis, Janssen-Cilag, Immunomedics, MSD, Pfizer, Lilly, Novartis, Agendia, Pierre-Fabre, Gilead, and SeaGen outside the submitted work. S. Schmidt reports grants from AGO Studiengruppe GmbH during the conduct of the study. K. Prieske reports personal fees from GSK, AstraZeneca, Roche, Clovis Oncology, and Molecular health outside the submitted work. N. de Gregorio reports personal fees and non-financial support from Roche and personal fees from AstraZeneca, GSK, MSD, and Clovis outside the submitted work. J. Hauke reports grants from AGO Research GmbH during the conduct of the study. A. du Bois reports personal fees from AstraZeneca, GSK/Tesaro, Roche, Genmab/Seattle Genetics, Zodiac, BIOCAD, and Clovis outside the submitted work. L.F. Wessels reports grants from Genmab BV outside the submitted work. E. Hahnen reports grants from AGO Research GmbH during the conduct of the study as well as other support from AstraZeneca outside the submitted work; in addition, E. Hahnen has a patent for *BRCA1/2* classifiers pending. P. Harter reports grants from AstraZeneca during the conduct of the study as well as grants and personal fees from Immunogen and Clovis; personal fees from Stryker, Zai Lab, and MSD; and grants from Genmab, European Union, DKH, and DFG outside the submitted work. S.C. Linn reports grants from Starz Foundation and BBMRI-NL during the conduct of the study as well as grants and non-financial support from AstraZeneca, Genentech-Roche, Novartis, and Tesaro (now owned by GSK); grants from Eurocept-pharmaceuticals, Immunomedics; and other support from Daiichi-Sankyo and IBM outside the submitted work; in addition, S.C. Linn has a patent for *BRCA*-like ovarian cancer classifiers pending. R.K. Schmutzler reports grants from Ago Ovar study group during the conduct of the study as well as personal fees from AstraZeneca outside the submitted work. No disclosures were reported by the other authors.

Authors' Contributions

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