Gynecologic Oncology 171 (2023) 106–113

Contents lists available at ScienceDirect

Gynecologic Oncology

journal homepage: www.elsevier.com/locate/ygyno

A RAD51 functional assay as a candidate test for homologous recombination deficiency in ovarian cancer

Félix Blanc-Durand [a], Elisa Yaniz-Galende [b], Alba Llop-Guevara [c], Catherine Genestie [d], Violeta Serra [c], Andrea Herencia-Ropero [c], Christophe Klein [e], Dominique Berton [f], Alain Lortholary [g], Nadine Dohollou [h], Christophe Desauw [i], Michel Fabbro [j], Emmanuelle Malaurie [k], Nathalie Bonichon-Lamaichhane [l], Coraline Dubot [m], Jean Emmanuel Kurtz [n], Gaëtan de Rauglaudre [o], Nadia Raban [p], Annick Chevalier-Place [q], Gwenael Ferron [r], Marie-Christine Kaminsky [s], Claire Kramer [t], Etienne Rouleau [u], Alexandra Leary [a,b,*]

[a] Medical Oncology, Gynecology Unit, Gustave Roussy Institute, Villejuif, France
[b] INSERM UMR981, Gustave Roussy Institute, Villejuif, France
[c] Experimental Therapeutics Group, Vall d'Hebron Institute of Oncology, Barcelona, Spain
[d] Pathology Department, Gustave Roussy Institute, Villejuif, France
[e] Center of Cellular Imaging and Cytometry, INSERM UMRS 1138, Cordeliers Research Center, Paris, France
[f] Medical Oncology, GINECO & Institut de Cancérologie de l'Ouest, Saint-Herblain, France
[g] Medical Oncology, GINECO-Hôpital Privé du Confluent, Nantes, France
[h] Medical Oncology, Polyclinique Bordeaux Nord Aquitain, Bordeaux, France
[i] Medical Oncology, Centre Hospitalier Universitaire, Lille, France
[j] Medical Oncology, ICM Val d'Aurelle, Montpellier, France
[k] Medical Oncology, Centre Hospitalier Intercommunal de Créteil, Créteil, France
[l] Medical Oncology, Clinique Tivoli-Ducos, Bordeaux, France
[m] Medical Oncology, GINECO and Institut Curie - Hôpital René Huguenin, Saint-Cloud, France
[n] Medical Oncology, CHU Stasbourg, Strasbourg, France
[o] Medical Oncology, GINECO and Institut Sainte- Catherine, Avignon, France
[p] Medical Oncology, GINECO and CHU La Milétrie, Poitiers, France
[q] Medical Oncology, Centre Oscar Lambret, Lille, France
[r] Medical Oncology, GINECO and Institut Claudius Regaud, Toulouse, France
[s] Medical Oncology, GINECO and Institut de Cancérologie de Lorraine, Vandoeuvre-Les-Nancy, France
[t] Department of Pathology, Leiden University Medical Center, Leiden, the Netherlands
[u] Cancer Genetics Laboratory, Gustave Roussy Institute, Villejuif, France

HIGHLIGHTS

- RAD51 functional assay requires minimal tissue and yields contributive results in >90% of cases.
- This assay identifies 54% of EOC patients with RAD51-low tumors and these patients presented better outcome.
- Among BRCA-mutated tumors, RAD51-high tumors showed poorer response to neoadjuvant chemotherapy

ARTICLE INFO

Article history:
Received 3 October 2022
Received in revised form 16 January 2023
Accepted 20 January 2023
Available online 1 March 2023

Keywords:
Ovarian cancer
BRCA

ABSTRACT

Rationale. Homologous recombination deficiency (HRD), defined as BRCA1/2 mutation (BRCAmut) or high genomic instability, is used to identify ovarian cancer (OC) patients most likely to benefit from PARP inhibitors. While these tests are useful, they are imperfect. Another approach is to measure the capacity of tumor cells to form RAD51 foci in the presence of DNA damage using an immunofluorescence assay (IF). We aimed to describe for the first time this assay in OC and correlate it to platinum response and BRCAmut.

Methods. Tumor samples were prospectively collected from the randomized CHIVA trial of neoadjuvant platinum +/− nintedanib. IF for RAD51, GMN and gH2AX was performed on FFPE blocks. Tumors were considered RAD51-low if ≤10% of GMN-positive tumor cells had ≥5 RAD51 foci. BRCAmut were identified by NGS.

* Corresponding author at: Medical Oncology Department, Gynecology Unit, Lead Translational Research team, INSERM U981, Institut Gustave Roussy, 114 rue Édouard Vaillant, 94800 Villejuif, France.
E-mail address: alexandra.leary@gustaveroussy.fr (A. Leary).

https://doi.org/10.1016/j.ygyno.2023.01.026

Homologous recombination
RAD51
Neoadjuvant chemotherapy

Results. 155 samples were available. RAD51 assay was contributive for 92% of samples and NGS available for 77%. gH2AX foci confirmed the presence of significant basal DNA damage. 54% of samples were considered HRD by RAD51 and presented higher overall response rates to neoadjuvant platinum ($P = 0.04$) and longer progression-free survival ($P = 0.02$). In addition, 67% of BRCAmut were HRD by RAD51. Among BRCAmut, RAD51-high tumors seem to harbor poorer response to chemotherapy ($P = 0.02$).

Conclusions. We evaluated a functional assay of HR competency. OC demonstrate high levels of DNA damage, yet 54% fail to form RAD51 foci. These RAD51-low OC tend to be more sensitive to neoadjuvant platinum. The RAD51 assay also identified a subset of RAD51-high BRCAmut tumors with unexpected poor platinum response.

1. Introduction

Epithelial ovarian cancer (EOC) affects around 300,000 patients worldwide and represents the 5th cause of women mortality in developed countries [1]. Platinum-based chemotherapy is the cornerstone of initial medical management whether as adjuvant treatment after complete cytoreductive surgery or as neoadjuvant treatment for inoperable disease [2,3]. Maintenance with bevacizumab results in a modest improvement in progression-free survival for women with advanced EOC [4,5]. Nevertheless, a large majority of patients develop refractory disease within the next few years.

Recently, the development of poly(adenosine diphosphate–ribose) polymerase (PARP) inhibitors have transformed the 1st line EOC treatment landscape, bringing unprecedented benefit for patients. The SOLO1 trial demonstrated tremendous improvement in disease-free survival of *BRCA*-mutated tumors treated with olaparib as 1st line maintenance after standard platinum-based chemotherapy [6]. A benefit was also observed in *BRCA*-wild-type (WT) EOC treated with niraparib, and in *BRCA*-WT cancers with a high genomic instability score (GIS) according to Myriad® testing treated with olaparib in combination with bevacizumab, as frontline maintenance after chemotherapy [7,8].

Approximately 25% of patients with EOC harbor germline or somatic *BRCA* 1 or 2 mutations and, considering the benefit of PARP inhibitors in this population, molecular assays have entered routine practice, regardless of family history. An additional 20% have BRCA-WT tumors but classified as homologous recombination deficient (HRD) on the basis of a high GIS and derive comparable benefit from frontline PARP inhibition [7,9].

While these genomic tests (*BRCA* mutation and GIS) are useful, they are imperfect. A subset of *BRCA*-mutated EOC demonstrate PARPi resistance, and some patients without *BRCA* mutation and a low GIS also benefited from niraparib compared to placebo in the PRIMA trial [8]. In addition, these genomic tests require a significant amount of tumor tissue and result in a non-contributive result in 15% of patients due to low cellularity or poor quality DNA [7,8]. Finally, GIS measure genomic "scars" resulting from HRD, as such they are static and will not evolve or change in the event of secondary events restoring HR proficiency. Therefore, additional biomarkers are urgently needed to better identify real-time homologous recombination proficiency status [10].

In this regard, functional HR assays are emerging as candidate biomarkers. RAD51 is a key downstream effector of the HR pathway, it mediates the homology search of the single-strand DNA and initiates strand invasion [11]. Numerous assays have been developed to detect RAD51 foci formation in response to DNA damage as a reflection of HR competency [12–16]. This approach measures the capacity of tumor cells to recruit nuclear RAD51 foci during S/G2 phase in the presence of extensive double strand DNA damage. Initially these assays required fresh tissue and exogenous DNA damage. However, certain tumors such as triple negative breast or high grade ovarian cancers may harbor high levels of basal endogenous double strand DNA breaks and thus may not require exogenous DNA damage. In this regard, Castroviejo-Bermejo et al. demonstrated that the evaluation of RAD51 foci in fixed treatment naïve tumors predicted PARPi benefit in breast cancer patient-derived xenografts and was feasible in archival patient samples [17–19].

We aimed to evaluate for the first time the multiplexed immunefluorescent RAD51 assay in untreated formalin-fixed paraffin embedded (FFPE) EOC samples from patients treated in a prospective trial of neoadjuvant platinum chemotherapy and correlate RAD51 status to platinum response and BRCA mutations.

2. Material and methods

2.1. Patients

The CHIVA trial (ClinicalTrials.gov identifier: NCT01583322) recruited patients with inoperable FIGO IIIC/IV EOC who were candidate to receive neoadjuvant chemotherapy (NACT). Between January 2013 and May 2015, 188 patients were randomized to 3 cycles of carboplatin AUC5 and paclitaxel 175 mg/m2 with nintedanib (BIBF) or placebo before debulking surgery, followed by adjuvant chemotherapy with or without nintedanib. Per protocol, formalin-fixed paraffin-embedded (FFPE) tumor samples from diagnostic laparoscopy were collected and 155 samples containing tumor cells were prospectively collected and centralized in an academic tumor bank. All samples involved distant localizations (peritoneum).

Response rates at the end of NACT were assessed according to RECIST1.1 and were categorized in two groups: objective response (OR+) when partial response or complete response was observed as best response, and no objective response (OR−) when stability or progressive disease was observed as best response. In addition, the achievement of complete cytoreduction (CC0) at interval debulking surgery was also assessed. Both OR+ and CC0 were independent factors associated with improved outcomes [20].

The trial was negative: the addition of nintedanib resulted in a statistically significant reduction in PFS and objective response, attributable to increased toxicity and lower chemotherapy exposure [21]. Thus, correlations between RAD51 assay and baseline characteristics were evaluated in the intent to treat population (ITT), but correlations with platinum response in the placebo group (P) only.

The methods were performed in accordance with relevant guidelines and regulations and were authorized by ANSM on June 6, 2012 and received a favorable opinion of the local medical ethics committee Ile de France 1 on March 29, 2012. All the patients provided written consent to translational research substudies.

2.2. RAD51 functional assay

Each FFPE tumor sample was reviewed by the same expert pathologist who circled tumor rich areas. Tissue microarrays (TMA) were then constructed from 3x1mm tumor cores. Immunofluorescence for nuclear RAD51, geminin (S/G2 phase marker), γH2AX (double strand DNA damage marker) and DAPI was performed on 3 μm-thick FFPE sections from these TMAs. In the event that there was insufficient tumor cellularity on the three spots from the TMA, a 3 μm section from the

corresponding whole block was used. The immunofluorescence staining was performed as previously described [17–19]. Briefly, all sections were heated at 60 °C for 1 h, deparaffinized with xylene and rehydrated with decreasing concentrations of ethanol. For target antigen retrieval, sections were pre-heated in DAKO Antigen retrieval buffer (pH 9.0) in a microwave for 20 min. Then, sections were cooled down for 30 min at room temperature (RT), incubated in distilled water for 5 min, permeabilized with DAKO Wash Buffer for 5 min and incubated in blocking buffer (1% bovine serum albumin diluted in DAKO Wash Buffer) for 5 min. Subsequently, sections were incubated with primary antibodies (diluted in DAKO Antibody Diluent) for 1 h at room temperature, washed (DAKO Wash Buffer) for 5 min and incubated in blocking buffer for 5 min. Finally, sections were incubated with secondary antibodies (diluted in blocking buffer) for 30 min at RT, washed for 5 min, incubated in blocking buffer for 5 min and incubated in distilled water for 5 min. Sections were dehydrated with increasing concentrations of ethanol and mounted with DAPI ProLong Gold antifading reagent. Biomarker scoring was manual and life, and immunofluorescence images were acquired using Olympus DP72 microscope.

Geminin, a cell-cycle regulator that prevents DNA replications was used as counterstaining to mark for S/G2-cell cycle phase. RAD51 foci were quantified on TMA and patient tumor whole sections, by scoring the percentage of geminin-positive tumor cells with ≥5 RAD51 foci per nucleus. Scoring was blindly performed using a 60×/1.4 oil immersion lens. One hundred geminin-positive cells from 4 to 5 representative areas of each sample were counted. Tumors were classified as RAD51-low when ≤10% Geminin^{+} tumor cells were RAD51 positive (≥5 RAD51 foci) (Fig. 1). This cut-off was based on a previously validated cut-off in breast cancer [14,17–19]. Two independent readers (EYG and ALG) scored the same first 30 samples and achieved great consistency, the rest of the cohort was scored by one reader (EYG).

The amount of DNA damage was quantified on all EOC tumor samples by scoring the percentage of geminin-positive cells with γH2AX foci, as described for RAD51 scoring. Confirmation of marker expression on tumor cells and not normal cells was performed by review of a sequential H&E stained slide.

2.3. Next generation targeted sequencing

The FFPE samples were extracted with the Maxwell Promega for the extraction of genomic DNA with the Maxwell® RSC DNA FFPE Kit (AS1450). The proteinase K treatment was done overnight at 56 °C and finalizing at 80 °C during 4 h. If DNA quantity or quality was poor, then DNA was extracted from interval debulking surgery samples after NACT.

The panel targets all tumor suppressors or oncogenes covering the complete sequencing of the codifying region of a total of 411 genes identifies point mutations, including single-nucleotide variants and small indels. The detection of CNVs throughout the genome, from gains and losses of chromosomes or complete chromosomal arms, to specific genes and even exons was based on the coverage ratio. The total size of this panel is 1,739,310-pb. The custom panel uses the SureSelect XT HS kit (Agilent Technologies, Santa Clara, CA United States) designed for small amounts of FFPE DNA as input and detect low allelic frequencies. After the enzymatic fragmentation of 50–200 ng of tumoral gDNA from each sample to an average of 200–400 bases The full preparation was done on a Bravo equipment option B (Agilent). Library quality control was performed on Tapestation 2000 and the commercial kits D1000 reagents and D1000 ScreenTape (Agilent). DNA samples were end-repaired, dA-tailed and ligated to the molecular-barcoded adaptor system and purified using AMPure XP beads. Pooled libraries containing captured DNA fragments were subsequently sequenced on an NovaSeq 6000 platform (Illumina) as 2 × 150-bp paired-end reads. Sequences were demultiplexed using an in-house tool.

The data analysis pipeline included the following algorithms developed internally: BWA-MEM v-0.7.12 for read alignment to the hg19 human reference genome and Samtools v-1.2 and Picard-tools v-1.139 for PCR duplicate quantification and removal. GATK Haplotype v-3.4-46, snpEff v-4.0 and MutaCaller-1.7 (home pileup internally developed) were used for variant calling and classification. Variants were called with a minimum allelic frequency threshold of 1% for already classified variants (those known in the internal database) and 5% for non-classified variants, and a read depth threshold of 30× for the total reads at the variant location and at least 10× for the variant.

Several filters were applied to further select for potential relevant variants among the called variants. The population databases Exac and gnomAd were used to automatically filter out polymorphism as soon as the population frequency was higher than 0.5%. Non-classified variants (not known in the internal database) were excluded if the intrarun recurrency was superior to 4 [9]. The in silico panel HR-gene includes *TM*, exons 2–63; *BARD1*, exons 1–10; *BRCA1*, exons 1–24; *BRCA2*, exons 2–27; *BRIP1*, exons 2–20; *CDK12*, exons 1–14; *CHEK2*, exons 2–15; *PALB2*, exons 1–13; *RAD51C*, exons 1–9; and *RAD51D*, exons 1–14. Additional genes included in the panel were *TP53* (exons 1–12) and *CCNE1* (only for amplification detection).

Variants were categorized using the 5-tier pathogenicity classification according to Plon and colleagues, 2008; class 1 = benign, class 2 = likely benign, class 3 = variant of unknown significance (VUS), class 4 = likely pathogenic, and class 5 = pathogenic [22]. Only class 3, 4, and 5 variants are reported in the manuscript.

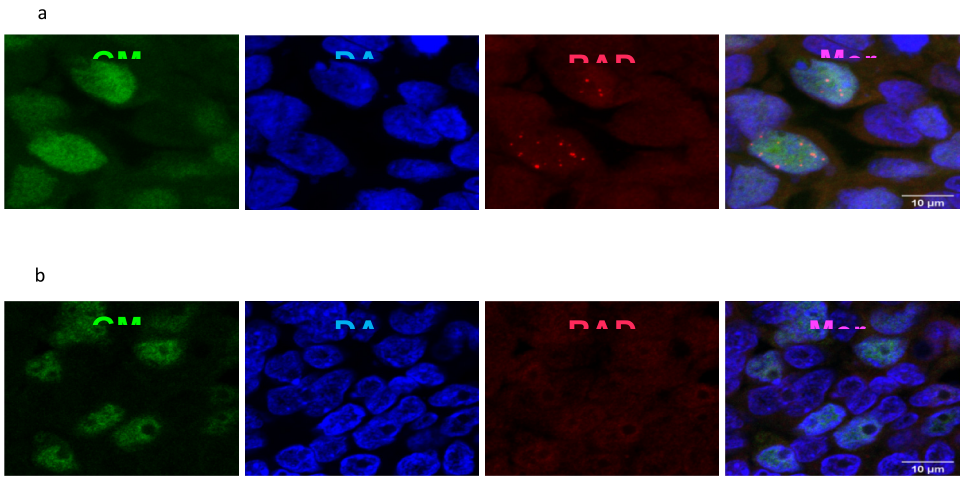

Fig. 1. (a) Representative image of a HR proficient tumor based on the presence of RAD51 nuclear foci (b) Representative image of a RAD51-low tumor (HRD). GMN = Geminin.

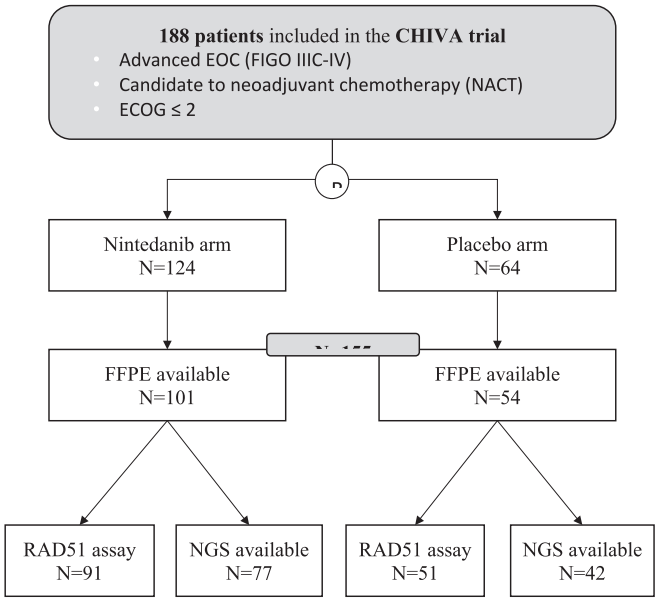

Fig. 2. Consort Diagram representing participant flow in the study.

The deletion and duplication of exons were defined with the average ratio of coverage for each exon or part of exon. A ratio under 0,5 was considered as deleted and a ratio superior to 1,3 was considered as duplicated. The full deletion of the gene was not considered with this approach, but the comparison between the neoplastic cells percentage and the allelic fraction was used to determine a putative loss of heterozygosity. If the allelic fraction represents the double of the neoplastic cells percentage, the LOH with a deletion of the allele was inferred.

In *BRCA* mutated tumors, SNP array was performed on FFPE DNA extracted samples with OncoScan FFPE Assay Kit (335 k probes, Thermo Fisher Scientific, Waltham, Massachusetts, United States) to confirm that the genomic event was bi-allelic on the *BRCA1* and *BRCA2* locus only.

2.4. Statistics

Statistical analysis was performed on the whole cohort; no formal calculation of power or sample size was needed. Descriptive statistics were used to summarize patient demographic and clinical characteristics.

Comparisons between categorical variables were performed using a non-parametric Fisher's exact test. A *P*-value of <0,05 was considered significant. Overall survival and disease-free survival were estimated using the method of Kaplan and Meier and presented with Rothman's 95% confidence intervals at 5 and 10 years. Hazard ratios with 95% confidence intervals were calculated using Cox models with no adjustment.

All statistical analyses were carried out using Prism (version 8.0) and RStudio (version 1.4.1103).

3. Results

3.1. Study population

155 patients with available FFPE material were analyzed in this study. 101 patients in the nintedanib group and 54 in the placebo (Fig. 2). The median age was 64 years, interquartile range (IQR) [59;69], with patients presenting good performance status (ECOG 0–1 in 89.4% of the cases). After centralized pathological review, a large majority of patients had serous/papillary histologic subtype (90%) and high grade (Grade 3) tumors (97%). Only 2 patients with Grade 1 tumors were included. 77% of patients presented FIGO stage III disease and 23% FIGO stage IV. Clinical characteristics were well balanced between the two subgroups and similar with the overall CHIVA cohort (Table 1 and Supplementary Table 1).

Median overall survival (mOS) was 37.9 months (95% CI 32.5–47.1) and median progression-free survival (mPFS) was 14.5 months (95% CI 13.3–16.7) (Supplementary Table 2). The nintedanib arm presented significantly poorer outcomes in comparison to the placebo subgroup both in OS, 34.3 months (95% CI 27.6–41) vs. 44.1 (95% CI 34.2 - not achieved [NA]) ($P = 0.04$), as well as in PFS, 13.7 (95% CI 11.7–15.4) vs. 17.2 months (95% CI 13.7–22.5) ($P = 0.03$).

Table 1
Patients characteristics and response to neoadjuvant chemotherapy (NACT) for epithelial ovarian cancer with available FFPE material in the CHIVA trial. Med = median; IQR = interquartile range; *BIBF versus placebo according to Fisher's exact test.

Variable		Total $N = 155$	BIBF $N = 101$	Placebo $N = 54$	*P*-value*
Age	Med [Range]	64.0 (31–79)	64.0 (31–79)	64.0 (43–79)	0.59
Histology (Central pathologic review)	Serous/papillary	109 (89%)	70 (90%)	39 (87%)	0.59
	Endometrioid	1 (1%)	1 (1%)	0 (0%)	
	Carcinosarcoma	3 (2%)	1 (1%)	2 (4%)	
	Clear Cell	1 (1%)	1 (1%)	0	
	Undifferenciated	9 (7%)	5 (5%)	4 (9%)	
	Unknown	52	36	16	
Grade	1	2 (2%)	2 (3%)	0 (0%)	0.68
	3	86 (98%)	58 (97%)	28 (100%)	
	Missing	67	41	26	
FIGO stage at diagnostic	IIIC	119 (77%)	80 (79%)	39 (72%)	0.33
	IV	36 (23%)	21 (21%)	15 (28%)	
ECOG	0–1	138 (90%)	92 (93%)	46 (85%)	0.16
	2	15 (10%)	7 (7%)	8 (15%)	
	Missing	2	2	0	
Response rate to NACT	Yes	57 (43%)	30 (36%)	27 (55%)	**0.04**
	No	75 (57%)	53 (64%)	22 (45%)	
	Missing	23	18	5	
Complete cytoreductive surgery	Yes	77 (52%)	44 (46%)	33 (64%)	**0.04**
	No	70 (48%)	52 (54%)	18 (35%)	
	Missing	8	5	3	

Table 2
List of molecular alterations in TP53 and BRCA genes detected by in-house panel NGS. WT = wild-type; *BIBF versus placebo according to Fisher's exact test.

Variable		Total $N = 155$	BIBF $N = 101$	Placebo $N = 54$	*P*-value*
TP53 status	TP53 mutation	104 (87%)	66 (86%)	38 (90%)	0.83
	TP53 intronic mutations	7 (6%)	5 (6%)	2 (5%)	
	WT	8 (7%)	6 (8%)	2 (5%)	
	Missing	36	24	12	
BRCA status	*BRCA1* Mutation	10 (8%)	4 (5%)	6 (14%)	0.30
	BRCA2 Mutation	8 (7%)	4 (5%)	4 (10%)	
	BRCA1 deletion	2 (2%)	2 (3%)	0	
	BRCA2 deletion	4 (3%)	3 (4%)	1 (2%)	
	WT	95 (80%)	64 (83%)	31 (74%)	
	Missing	36	24	12	

3.2. Genomic alterations

119 samples had acceptable DNA quantity and quality for NGS resulting in a contributive testing rate of 77% (Fig. 2 and Table 2). As expected, the prevalence of *TP53* mutations was high, found in >93% (111/119) of the tumors, with a majority of single-nucleotide variation (SNV) mutations. 8 tumors did not exhibit any *TP53* mutation (6 in the nintedanib group and 2 in the placebo group), however, all of them were high grade tumors (grade 3).

Deleterious *BRCA* SNV mutations (*BRCAmut*) were detected in 18 out of the 119 tumors (15%), with 10 *BRCA1* and 8 *BRCA2* mutations. In addition, 6 *BRCA* deletions (*BRCAdel*) were detected (5.0%).

3.3. DNA damage and prevalence of HRD by RAD51

This cohort of untreated EOC samples demonstrated high levels of γH2AX foci with a median value of 86% of tumor cells (TC) being γH2AX-positive per sample and IQR of [56.47–100], thus testifying to high levels of spontaneous DNA damage (Fig. 3A, B). Among the small subset of tumors with low γH2AX expression (<10% TC positive) ($N = 8$), all were centrally confirmed as high-grade serous/papillary tumors and TP53-mutated tumors and thus were not excluded from the analyses.

Out of the overall 155 patients from the CHIVA cohort with available tissue, RAD51 assay was assessable in 142 patients resulting in a contributive test result of 92% (Fig. 2). Median RAD51 score (percent of geminin+ TC also RAD51+) was 8% with IQR [1.7–25.6], 76 tumors (54%) were scored RAD51-low (score ≤ 10%) (Fig. 4). The proportion of RAD51-low tumors was numerically higher in the placebo than in the nintedanib arm, however this difference was not statistically significant (63% vs 49% for P vs N; $P = 0.11$).

Non-serous/papillary tumors (including clear-cell, endometrioïd, carcinosarcomas and undifferentiated tumors) were RAD51-high in 6 out of the 12 analyzable cases (50%). TP53-WT tumors were considered RAD51-high in 3 out of the 7 analyzable cases (43%).

3.4. RAD51 correlation with BRCA alterations

Among *BRCAmut* tumors, 67% (12/18) were scored RAD51-low: 5/10 (50%) of *BRCA1* mutated tumors vs. 7/8 (88%) *BRCA2* mutated tumors (Fig. 4, Supplementary Fig. 1). All 6 tumors harboring *BRCA* deletions were classified as RAD51-low.

Six tumors with confirmed *BRCA* mutations presented high RAD51 expression. All of them were grade III, serous/papillary and TP53-mutated tumors. In one patient (ID 2), the RAD51 score was just above the cut-off (RAD51 score = 11%). The 5 remaining tumors (ID 62, 66, 91, 106, 151) were characterized by SNP array. All 5 patients presented complete loss of heterozygosity (chromosome 17 or 13 deletion associated with isodisomia in case of BRCA1 or BRCA2 deficiency respectively), confirming bi-allelic events (Supplementary Fig. 2).

3.5. RAD51 correlation with clinical outcomes

We then correlated RAD51 status to platinum responsiveness in the placebo subgroup. 27 patients in the placebo arm presented partial or complete response to neoadjuvant platinum chemotherapy (55%) whereas 22 did not (45%), 33 patients benefited from CC0 surgery (68.8%), 18 did not (31.2%). Patients with RAD51-low EOC presented significantly higher overall response rates to neoadjuvant chemotherapy (68% vs 37%, RAD51-low vs RAD51-high; $P = 0.04$). Likewise, patients with RAD51-low tumors presented numerically higher complete cytoreductive surgery rates after NACT, however the difference did not reach statistical significance (76% vs 52%, $P = 0.13$) (Supplementary table 3). In the placebo subgroup, median PFS was 14.1 months (95% CI 12.4–21.4) in the RAD51-high subgroup vs. 18.0 months (95% CI 13.7-NA) in the RAD51-low subgroup, $P = 0.03$. Likewise, median OS was 32.7 months (95% CI 25.2-NA) vs. NA (95%CI 39.7-NA) in the RAD51-high vs-low subgroup respectively, $P = 0.06$. (Fig. 5).

Among *BRCA* mutation carrier patients, response rate to NACT was 67%. Interestingly, in this small exploratory subset of patients, RAD51-

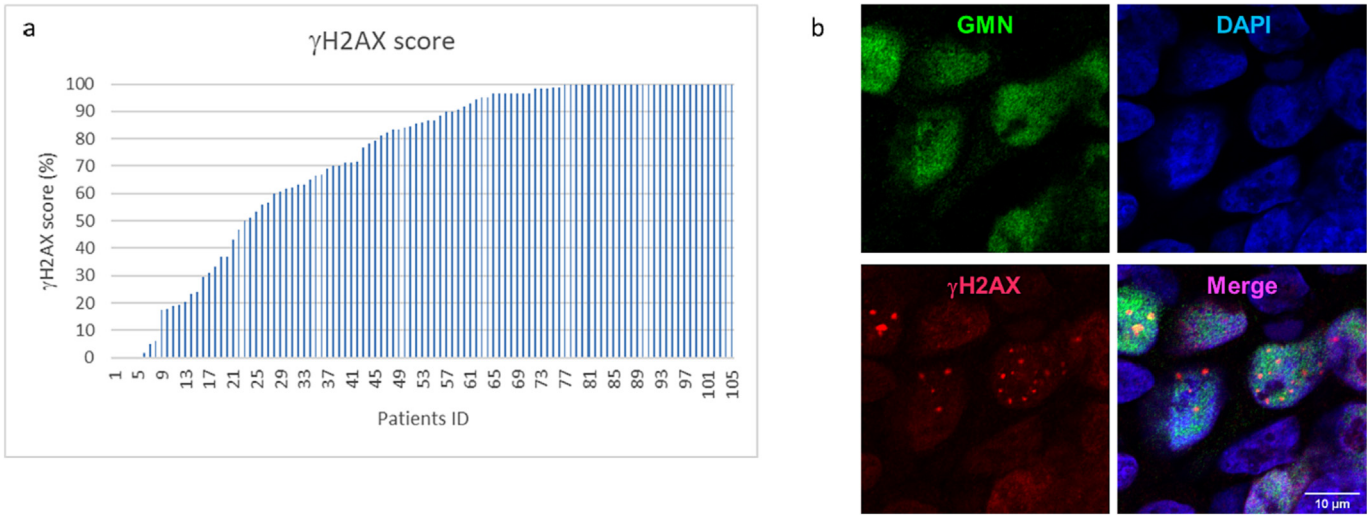

Fig. 3. (a) Distribution of gH2AX foci. Median 86.65; IQR of [56.47–100]. 8 patients with low gH2AX (<10%), among them two patients with low-grade tumors. (b) Representative image of a tumor with dsDNA damage based on the presence of gH2AX nuclear foci.

Figure 4

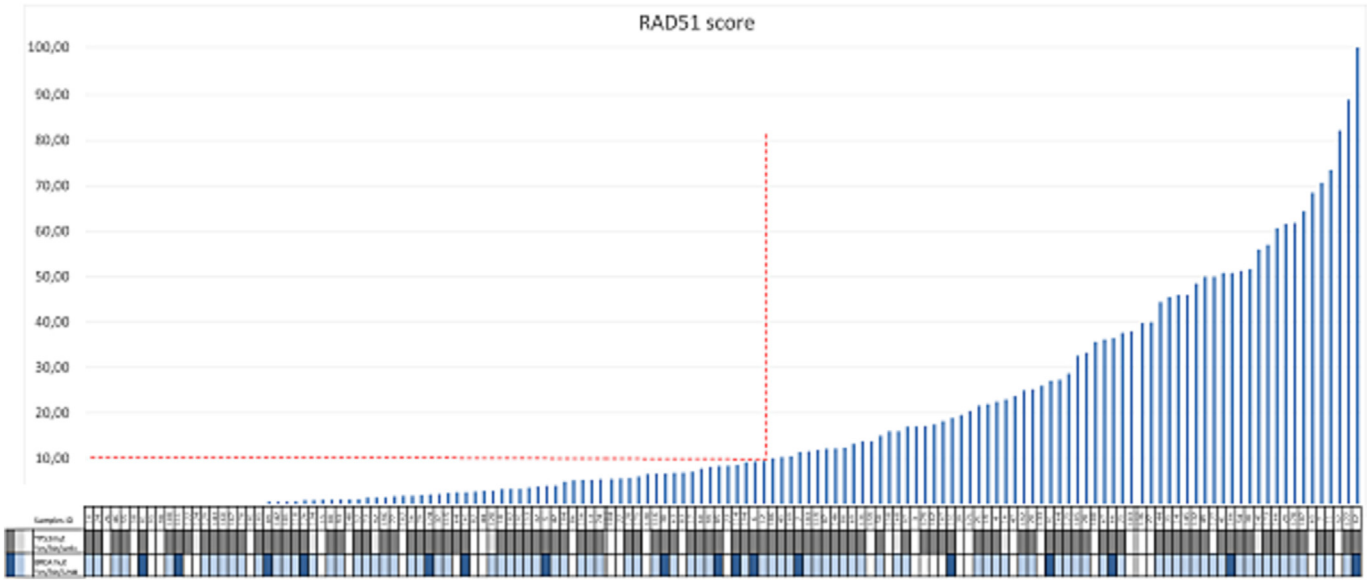

Fig. 4. Distribution of RAD51 scoring ($N = 142$). Median 8.35; IQR [1.73–25.63]. 76 patients (53.5%) were considered RAD51 low (score ≤ 10). ID correspondence with TP53mut and BRCAmut

high tumors presented worst response rates to NACT (RR 17% versus 77% among RAD51-high vs RAD51-low, $P = 0.04$). However, we did not observe any significant difference in complete cytoreductive surgery rates, PFS or OS.

4. Discussion

We present an exploratory study testing a novel RAD51 IF assay in a clinical trial-quality cohort of ovarian cancer patients treated with neoadjuvant platinum chemotherapy. The assay requires very minimal tumor material (two FFPE sections 3 μm-thick), is feasible in TMA sections and yields contributive results in >90% of patients. In contrast, genomic testing which requires more tumor material and >100 ng DNA results in 80% contributive tests [7,8]. In order to reliably evaluate HR deficiency, the assay is based on the failure to form nuclear RAD51 foci during S/G2 cell phase in the presence of double strand DNA damage. As expected, treatment naïve high grade ovarian tumors harbor high levels of spontaneous DNA damage, confirmed by γH2AX foci, and thus represent an ideal candidate to test HR functional tests on fixed basal tissue. Overall, more than half of the patients (54%) are considered

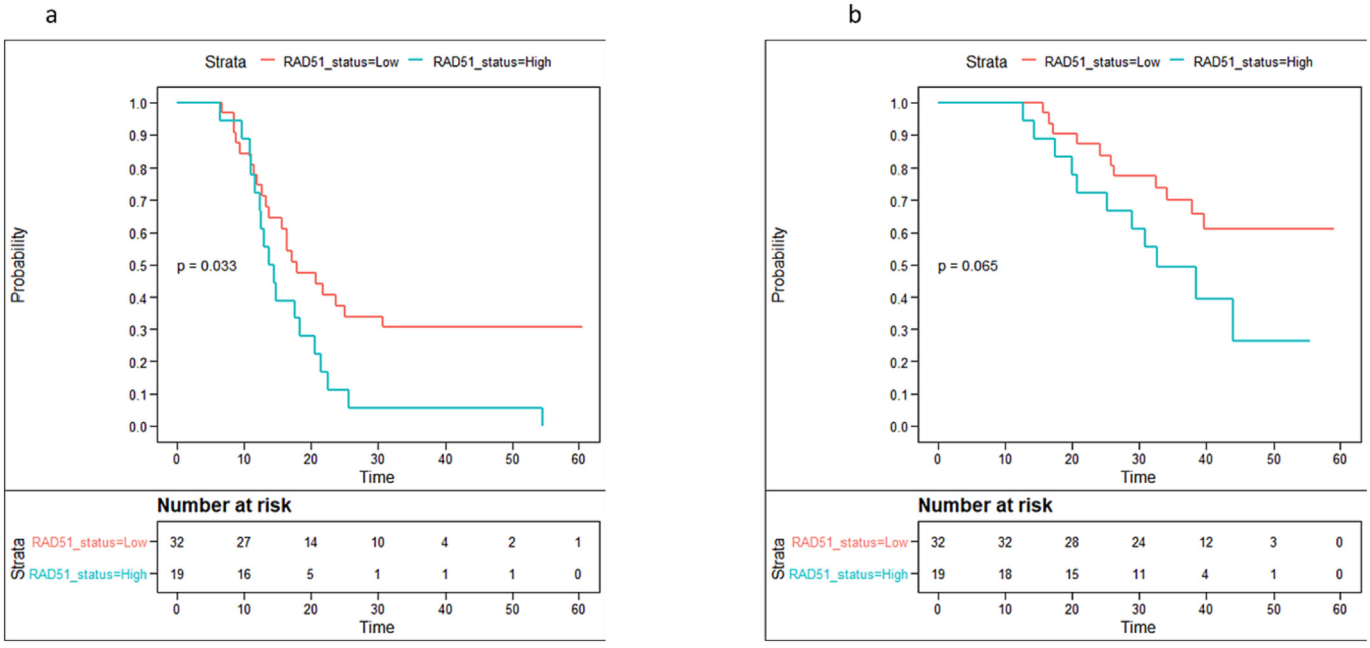

Fig. 5. [a] Progression free survival in the placebo subgroup ($N = 50$) based on the RAD51 score, $P = 0.033$. [b] Overall survival in the placebo subgroup (N = 50) based on the RAD51 score, $P = 0.065$.

RAD51-low and potentially present homologous recombination deficient tumors [18].

RAD51 status is imperfectly related to *BRCA* mutations, with a calculated accuracy of 53%. However, RAD51 assay identifies 66% of BRCAmut tumors as harboring functional HRD (RAD51-low). We can hypothesize that RAD51 assay has the potential to detect a BRCAmut subpopulation with primary resistance to platinum-based chemotherapy and probably HR proficiency. Although the exploratory nature of this study and the relatively small number of cases hamper definitive conclusions, we observed an intriguing correlation of HRD status by RAD51 status in neoadjuvant treatment outcome among *BRCAmut* tumors (ORR = 77% vs 17%; $P = 0.04$). A greater proportion of *BRCA2*mut than *BRCA1*mut tumors are HRD by RAD51. Once again, this result must be interpreted with caution regarding the respective number of patients involved ($N = 18$). However, this observation is consistent with reports suggesting that *BRCA2*m HGOC present better platinum response and outcome than *BRCA1*mut HGOC [24–26]. Our results are also in line with data from randomized PARPi trials showing that *BRCA2*mut HGOC tumors derive greater benefit from PARPi in the maintenance setting than *BRCA1*mut HGOC tumors [27]. Finally, in patient-derived xenograft models of *BRCA1/2* mutated breast cancer, non-HRD by RAD51 tumors were resistant to PARPi treatment ex vivo, despite confirmed deleterious *BRCA* mutation and LOH [18]. Taken together, these data suggest that the capacity of RAD51 assay to detect chemoresistant BRCA mutated tumors, unlikely to benefit from PARP inhibition, is worthwhile to be confirmed on larger cohorts.

We acknowledge the exploratory nature of our study responsible for several limitations. Firstly, RAD51 cut-off was defined based on preclinical, or clinical studies in breast cancer and may not be the optimal cut-off in patients' samples with HGOC [19]. Regarding the assay, the distribution of RAD51 scoring in HGOC is continuous rather than bi-modal, making the biologically relevant threshold hard to define. At the time the study was designed, we didn't have access to the Myriad HRD test, which represents the “gold standard” based on randomized phase III trials [7,8]. The correlation between RAD51 score and other available HRD tests is needed. We plan to complete our analyses with SNP array to confirm that detected *BRCA* deletions are biallelic events, as well as *BRCA1* and *RAD51* methylation status, found in around 10% of OC patients [28], which may explain the HRD status in a subgroup of *BRCA*-wt tumors [28,29]. Finally, the multiplicity of statistical tests and the subgroup analysis performed on the placebo cohort only might have biased our results and raise the hypothesis of false positive associations. Herein, our observations need further validation on independent cohorts.

To conclude, we report the performance of an assay of HR functionality in a homogenous cohort of advanced EOC. Around half of HGOC tumors are RAD51-low and present significantly improved outcome after neoadjuvant platinum. The RAD51 assay also identifies a small subset of RAD51-high *BRCA*mut tumors with poor platinum response. Whether this RAD51 functional assay may also predict PARP inhibitor benefit is currently being investigated.

Author contribution

- **Conceptualization:** FBD, ALe, EYG, ALG
- **Methodology:** FBD, EYG, ALG, VS, AHR, CK
- **Validation:** FBD, CG, EYG, CK, ER, ALe
- **Formal Analysis:** FBD, EYG, ER, ALe
- **Writing – Original draft:** FBD, ALe
- **Supervision:** CG, DB, ALo, ND, CD, MF, JEK, GDR, NR, ACP, GF, MCK
- **Funding acquisition:** EYG, ALG, ALe

All authors have read, revised and approved the final manuscript.

Financial support

ERA-NET Cofund (ERAPERMED 2019–215).

ALG received funding from Asociación Española Contra el Cáncer (AECC, INVES20095LLOP).

Aknowledgements

ARCAGY GINECO (https://www.arcagy.org/).

Data availability

The data generated in this study are available upon request from the corresponding author.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygyno.2023.01.026.

References

[1] F. Bray, et al., Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, CA Cancer J. Clin. 68 (2018) 394–424.
[2] A. Fagotti, et al., Survival analyses from a randomized trial of primary debulking surgery versus neoadjuvant chemotherapy for advanced epithelial ovarian cancer with high tumor load (SCORPION trial), J. Clin. Oncol. 36 (2018) 5516.
[3] I. Vergote, et al., Neoadjuvant chemotherapy or primary surgery in stage IIIC or IV ovarian Cancer, N. Engl. J. Med. 363 (2010) 943–953.
[4] R.A. Burger, et al., Incorporation of bevacizumab in the primary treatment of ovarian Cancer, N. Engl. J. Med. 365 (2011) 2473–2483.
[5] T.J. Perren, et al., A phase 3 trial of bevacizumab in ovarian Cancer, N. Engl. J. Med. 365 (2011) 2484–2496.
[6] K. Moore, et al., Maintenance Olaparib in patients with newly diagnosed advanced ovarian Cancer, N. Engl. J. Med. 379 (2018) 2495–2505.
[7] I. Ray-Coquard, et al., Olaparib plus bevacizumab as first-line maintenance in ovarian Cancer, N. Engl. J. Med. 381 (2019) 2416–2428.
[8] A. González-Martín, et al., Niraparib in patients with newly diagnosed advanced ovarian Cancer, N. Engl. J. Med. 381 (2019) 2391–2402.
[9] P.A. Konstantinopoulos, R. Ceccaldi, G.I. Shapiro, A.D. D'Andrea, Homologous recombination deficiency: exploiting the fundamental vulnerability of ovarian Cancer, Cancer Discov. 5 (2015) 1137–1154.
[10] E.M. Miller, et al., Utility of a custom designed next generation DNA sequencing gene panel to molecularly classify endometrial cancers according to the Cancer genome atlas subgroups, BMC Med. Genet. 13 (2020).
[11] M. Jasin, R. Rothstein, Repair of strand breaks by homologous recombination, Cold Spring Harb. Perspect. Biol. 5 (2013), a012740.
[12] M.M. Shah, et al., An ex vivo assay of XRT-induced Rad51 foci formation predicts response to PARP-inhibition in ovarian cancer, Gynecol. Oncol. 134 (2014) 331–337.
[13] T.G. Meijer, et al., Functional ex vivo assay reveals homologous recombination deficiency in breast Cancer beyond BRCA gene defects, Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. 24 (2018) 6277–6287.
[14] K.A.T. Naipal, et al., Functional ex vivo assay to select homologous recombination-deficient breast tumors for PARP inhibitor treatment, Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. 20 (2014) 4816–4826.
[15] P. ter Brugge, et al., Mechanisms of therapy resistance in patient-derived xenograft models of BRCA1-deficient breast Cancer, JNCI J. Natl. Cancer Inst. 108 (2016).
[16] M.M. de Jonge, et al., Frequent homologous recombination deficiency in high-grade endometrial carcinomas, Clin. Cancer Res. 25 (2019) 1087–1097.
[17] M. Castroviejo-Bermejo, et al., A RAD51 assay feasible in routine tumor samples calls PARP inhibitor response beyond BRCA mutation, EMBO Mol. Med. 10 (2018).
[18] C. Cruz, et al., RAD51 foci as a functional biomarker of homologous recombination repair and PARP inhibitor resistance in germline BRCA-mutated breast cancer, Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. 29 (2018) 1203–1210.
[19] A. Llop-Guevara, et al., Association of RAD51 with homologous recombination deficiency (HRD) and clinical outcomes in untreated triple-negative breast cancer (TNBC): analysis of the GeparSixto randomized clinical trial, Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. 32 (2021) 1590–1596.
[20] F. Lecuru, et al., Surrogate endpoint of progression-free (PFS) and overall survival (OS) for advanced ovarian cancer (AOC) patients (pts) treated with neo-adjuvant chemotherapy (NACT): results of the CHIVA randomized phase II GINECO study, Ann. Oncol. 30 (2019), v415.

[21] G. Ferron, et al., Impact of adding nintedanib to neoadjuvant chemotherapy (NACT) for advanced epithelial ovarian cancer (EOC) patients: the CHIVA double-blind randomized phase II GINECO study, J. Clin. Oncol. 37 (2019) 5512.
[22] S.E. Plon, et al., Sequence variant classification and reporting: recommendations for improving the interpretation of cancer susceptibility genetic test results, Hum. Mutat. 29 (2008) 1282–1291.
[24] D.M. Hyman, et al., Improved survival for BRCA2-associated serous ovarian cancer compared with both BRCA-negative and BRCA1-associated serous ovarian cancer, Cancer 118 (2012) 3703–3709.
[25] I. Cass, et al., Improved survival in women with BRCA-associated ovarian carcinoma, Cancer 97 (2003) 2187–2195.
[26] P. DiSilvestro, et al., Efficacy of maintenance Olaparib for patients with newly diagnosed advanced ovarian Cancer with a BRCA mutation: subgroup analysis findings from the SOLO1 trial, J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 38 (2020) 3528–3537.
[27] J. George, et al., Nonequivalent gene expression and copy number alterations in high-grade serous ovarian cancers with BRCA1 and BRCA2 mutations, Clin. Cancer Res. 19 (2013) 3474–3484.
[28] J. Veeck, et al., BRCA1 CpG Island Hypermethylation predicts sensitivity to poly (adenosine diphosphate)- ribose polymerase inhibitors, J. Clin. Oncol. 28 (2010) e563–e564.
[29] O. Kondrashova, et al., Methylation of all BRCA1 copies predicts response to the PARP inhibitor rucaparib in ovarian carcinoma, Nat. Commun. 9 (2018) 3970.