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The 8th Biennial Meeting of Asian Society of Gynecologic Oncology

J Gynecol Oncol. 2023 Jul;34(4):e45
https://doi.org/10.3802/jgo.2023.34.e45
pISSN 2005-0380·eISSN 2005-0399

JOURNAL OF GYNECOLOGIC ONCOLOGY

Original Article

RAD51/geminin/γH2AX immunohistochemical expression predicts platinum-based chemotherapy response in ovarian high-grade serous carcinoma

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Received: Sep 26, 2022
Revised: Dec 28, 2022
Accepted: Jan 18, 2023
Published online: Feb 3, 2023

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ABSTRACT

Objective: The RAD51 assay is a recently developed functional assay for homologous recombination deficiency (HRD) that reflects real-time HRD status. We aimed to identify the applicability and predictive value of RAD51 immunohistochemical expression in pre- and post-neoadjuvant chemotherapy (NAC) samples of ovarian high-grade serous carcinoma (HGSC).
Methods: We evaluated the immunohistochemical expression of RAD51/geminin/γH2AX in ovarian HGSC before and after NAC.
Results: In pre-NAC tumors (n=51), 74.5% (39/51) showed at least 25% of γH2AX-positive tumor cells, suggesting endogenous DNA damage. The RAD51-high group (41.0%, 16/39) showed significantly worse progression-free survival (PFS) compared to the RAD51-low group (51.3%, 20/39) (p=0.032). In post-NAC tumors (n=50), the RAD51-high group (36.0%, 18/50) showed worse PFS (p=0.013) and tended to present worse overall survival (p=0.067) compared to the RAD51-low group (64.0%, 32/50). RAD51-high cases were more likely to progress than RAD51-low cases at both 6 months and 12 months (p=0.046 and p=0.019, respectively). Of 34 patients with matched pre- and post-NAC RAD51 results, 44% (15/34) of pre-NAC RAD51 results were changed in the post-NAC tissue, and the RAD51 high-to-high group showed the worst PFS, while the low-to-low group showed the best PFS (p=0.031).
Conclusion: High RAD51 expression was significantly associated with worse PFS in HGSC, and post-NAC RAD51 status showed higher association than pre-NAC RAD51 status. Moreover, RAD51 status can be evaluated in a significant proportion of treatment-naïve HGSC samples. As RAD51 status dynamically changes, sequential follow-up of RAD51 status might reflect the biological behavior of HGSCs.

Keywords: Ovarian Cancer; RAD51 recombinase; Homologous Recombination; Neoadjuvant Chemotherapy; Immunohistochemistry

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Funding
This study was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (2021R1G1A1091264) and a faculty research grant from Yonsei University College of Medicine (6-2021-0244).

Conflict of Interest
No potential conflict of interest relevant to this article was reported.

Synopsis
High-RAD51 expression was significantly associated with worse progression-free survival in HGSC. RAD51 status can be evaluated in a significant proportion of γH2AX-positive treatment-naïve high-grade serous carcinoma (HGSC) tissues. Sequential follow-up of RAD51 status might reflect the biological behavior of HGSC.

INTRODUCTION

Ovarian cancer is the most fatal gynecological malignancy and the fifth leading cause of cancer-related death in women worldwide [1]. Among different types of ovarian cancer, high-grade serous carcinoma (HGSC) is the most common subtype with the worst prognosis, as it is usually diagnosed at an advanced stage [2,3]. Although the standard treatment for HGSC is debulking surgery with adjuvant chemotherapy, neoadjuvant chemotherapy (NAC) followed by interval debulking surgery has been increasing as a treatment option for patients with bulky advanced-stage HGSC [4]. After NAC, approximately 30% of patients have favorable progression-free survival (PFS), while the remaining 70% have high relapse rates [5,6].

Platinum chemotherapy is the cornerstone of chemotherapy for ovarian HGSC. Platinum compounds induce DNA double-strand breaks of the cells during replication, which are especially lethal to rapidly proliferating cancer cells. Homologous recombination (HR) is the most accurate pathway for repairing DNA double-strand breaks, and a significant proportion of cancers, especially breast and ovarian cancers, have HR deficiency (HRD), resulting in platinum sensitivity. Likewise, in inducing synthetic lethality, poly(ADP-ribose) polymerase (PARP) inhibitor is most effective in patients with HRD, and has become a game changer for ovarian cancer [7,8]. Approximately 50% of HGSCs exhibit HRD; thus, evaluation of the HRD status become utmost important for predicting treatment response of HGSC [9,10]. To assess the HRD status, genomic assays have been generally used; they detect mutations in HR repair genes (e.g., *BRCA1, BRCA2, ATM, RAD51*, and *FANCD2*) and genomic instability status by evaluating loss of heterozygosity, telomeric allelic imbalances, and large-scale state transitions [11,12]. However, genomic assays require complex interpretation, high cost, long turn-around time, and tissue with high tumor cellularity. Moreover, the genetic scar detected by genomic assays does not reflect the current functional HRD status of tumors [13].

In this regard, HRD functional assays targeting RAD51 protein expression have been developed [14-22]. RAD51 plays a central role in HR and is involved in a single downstream event in the HR-mediated DNA repair pathway [23]. As RAD51 is expressed only after DNA double-strand breaks in proliferating cells, early studies for RAD51 assays induced exogenous DNA damage prior to RAD51 evaluation, such as *ex vivo* irradiation, and used immunofluorescence (IF) multiplex staining to identify RAD51 foci in geminin (G2/S phase marker)-positive tumor cells. With the RAD51 IF assays, RAD51 status outperformed genomic test in predicting clinical outcomes and platinum/PARPi resistance in ovarian and breast cancers [14,16,19]. Furthermore, in breast cancers, good performance of the RAD51 assay has been reported in samples without prior exogenous DNA damage induction, which could be explained by endogenous DNA damage in tumor cells [22].

Subsequently, RAD51 assay using conventional immunohistochemistry (IHC) also showed a correlation with worse clinical outcomes and platinum resistance [18-21,24]. In RAD51

IHC assays, RAD51 expression was often evaluated as nuclear expression, as nuclear foci may be not distinct in IHC. However, the change in RAD51 expression before and after chemotherapy with RAD51/geminin/γH2AX co-evaluation has not been studied in HGSC, and the applicability of the RAD51 IHC assay in ovarian HGSC needs to be further investigated.

In this study, we evaluated the applicability and the predictive value of RAD51, geminin (G2/S phase marker), and γH2AX (DNA damage marker) using IHC in pre- and post-NAC RAD51 status in paired ovarian HGSC.

MATERIALS AND METHODS

1. Patients

A total of 220 patients were diagnosed with ovarian HGSC and received neoadjuvant platinum-based chemotherapy, followed by interval debulking surgery, between February 2012 and June 2020 at Yonsei Severance Hospital (Seoul, Korea). Of these, 54 cases with matched pre- and post-NAC ovarian tumor samples were available for the analysis. This study was approved by the institutional review board of Severance Hospital (IRB no. 4-2021-1391).

2. Histologic and conventional IHC analysis

All hematoxylin and eosin-stained tumor-containing ovarian tissue slides were independently reviewed. The chemotherapy response score (CRS) was evaluated according to a previous study [25]. Briefly, CRS 1 indicated no or minimal tumor response, CRS 2 indicated appreciable tumor response amid a viable tumor that is readily identifiable, and CRS 3 indicated complete or near-complete response with no residual tumor or nodules up to a maximum size of 2 mm.

Conventional IHC analysis was performed on whole sections of FFPE tumor tissue blocks. Four-μm-thick sections of surgically resected tissues were immunostained using a Ventana BenchMark XT system automated stainer (Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer's recommendations. The sections were incubated with antibodies against RAD51 (clone 14B4; GeneTex, Alton Pkwy Irvine, CA, USA), geminin (clone 10802-1-AP; ProteinTech Group, Chicago, IL, USA), and γH2AX (clone JBW301, Sigma-Aldrich, Dorset, UK). The detailed protocols are summarized in **Table S1**. After chromogenic visualization using an UltraView Universal DAB Detection Kit (Ventana Medical Systems), the slices were counterstained with hematoxylin, dehydrated in graded alcohols and xylene, and embedded in mounting solution. Appropriate positive and negative controls were concurrently stained to validate the staining method.

3. Interpretation of RAD51 status-related markers

As HR pathway is only active after DNA double-strand breaks in proliferating cells, we evaluated RAD51 after excluding low-γH2AX (DNA damage marker) or low-geminin (G2/S phase marker)-expressing tumor samples. In detail, after excluding tumors with low cellularity (less than 300 tumor cells per high-power field), tumors with less than 25% of γH2AX-positive cells and tumors with less than 40 geminin-positive cells per high power field were excluded according to a previous study [16]. Then, for RAD51 analysis, only nuclear expression was evaluated using the H-score (a semi-quantitative system with a total score range of 0–300). The percentage of positive cells (0–100%) was multiplied by the dominant intensity score of staining: 0, no appreciable staining; 1, barely detectable staining; 2, distinct

https://doi.org/10.3802/jgo.2023.34.e45

brown staining; and 3, strong dark brown staining. Applying the optimal cutoff for RAD51 expression determined based on maximally selected rank statistics formulated using the Contal and O'Quigley method, RAD51 H-score <20 was defined as RAD51-low and RAD51 H-score ≥20 was defined as RAD51-high [26]. Flow diagram for the sample selection and categorization, as well as the representative images of IHC staining for γH2AX, geminin, and RAD51, are shown in **Fig. 1**. All slides were evaluated by two experienced pathologists (E.P. and K.K.) in a blinded manner. If discrepancies occurred, a consensus was reached.

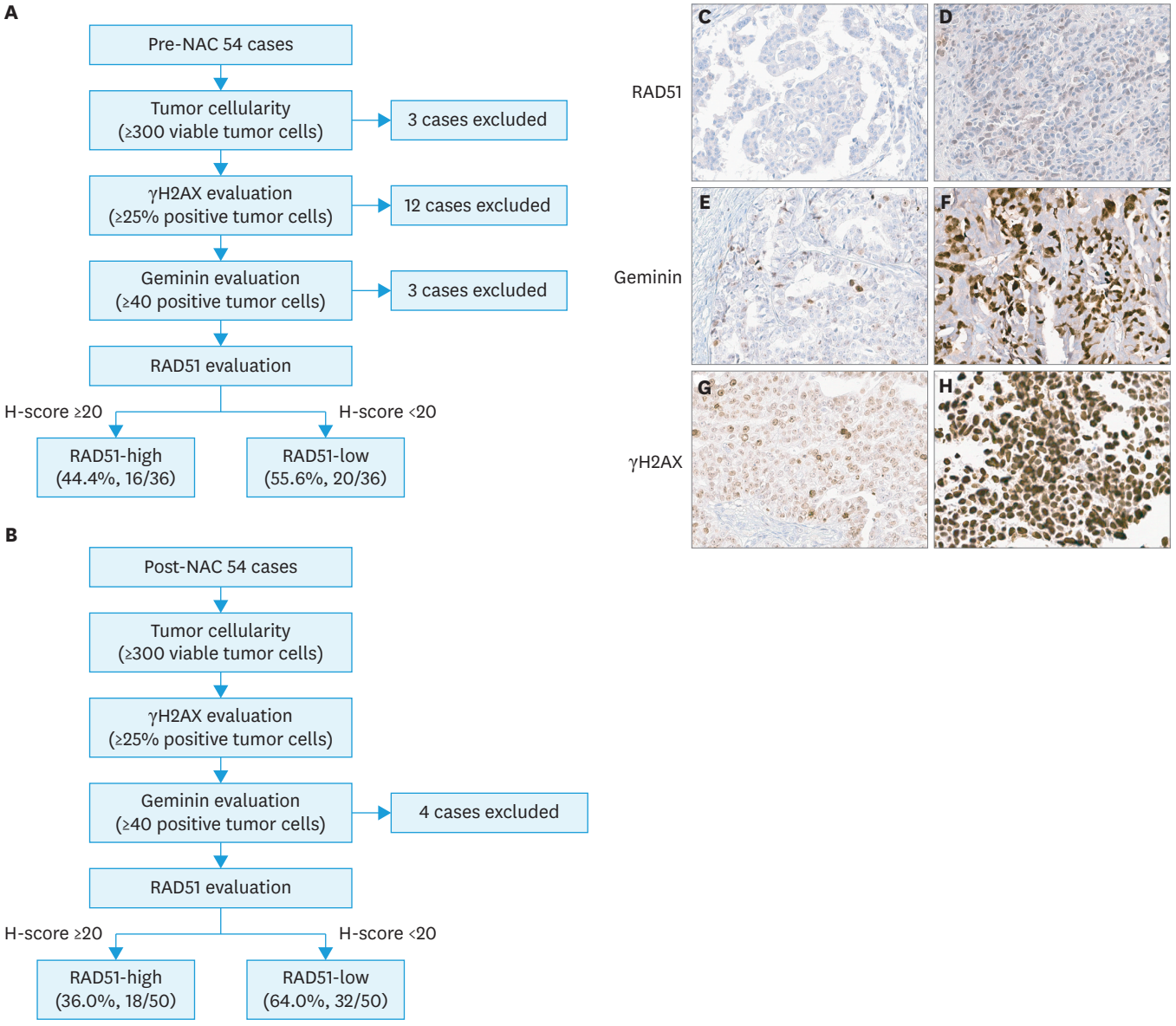

Fig. 1. Flow diagram for the sample selection and categorization in pre- and post- NAC samples and representative images of immunohistochemical staining for γH2AX, geminin, and RAD51 in ovarian high-grade serous carcinoma. (A) Flow diagram for pre-NAC samples. (B) Flow diagram for post-NAC samples. (C, D) γH2AX expression. (C) Negative (<25% in tumor cells). (D) Positive (≥25% in tumor cells). (E, F) Geminin expression. (E) Negative (<40 positive tumor cells). (F) Positive (≥40 positive tumor cells). (G, H) RAD51 expression. (G) Llow (H-score <20). (H) High (H-score ≥20).
NAC, neoadjuvant chemotherapy.

https://doi.org/10.3802/jgo.2023.34.e45

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4. Fluorescent multiplex IHC

For fluorescent multiplex IHC, 4-μm-thick sections of FFPE tissues were stained with RAD51 and geminin at prismCDX Co., Ltd. (Hwaseong, Korea). IF staining was performed on Leica Bond Rx™ Automated Stainer (Leica Biosystems, Newcastle, UK). The primary antibodies used included RAD51 (1:1000, clone 14B4; GeneTex) and geminin (1:1000, clone 10802-1-AP; ProteinTech Group), and Polymer HRP Ms+Rb (ARH1001EA; AKOYA Biosciences, MA, USA) was used as the secondary antibody. After the last step of antibody stripping, nuclei were subsequently visualized with DAPI, and the section was cover-slipped using ProLong Gold antifade reagent (P36934, Invitrogen, CA, USA). For RAD51 foci analysis, the percentage of geminin-positive cells with five or more RAD51 nuclear foci among all geminin-positive tumor cells was scored.

5. Germline *BRCA1/2* (gBRCA) genetic tests

gBRCA genetic testing was performed using genomic DNA from the peripheral blood samples. Sanger sequencing on a 3730 DNA Analyzer with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), followed by analysis using the Sequencher 5.3 software [27]. In a proportion of patients, next-generation sequencing using a custom panel, including *BRCA1* and *BRCA2* genes, was performed on a MiSeq sequencer (Illumina, San Diego, CA, USA) using the MiSeq Reagent Kit v2 (300 cycles). Bioinformatics analysis was performed using the Burrows-Wheeler Aligner, Genome Analysis Toolkit, Ensembl Variant Effect Predictor, and our custom pipelines. Final interpretation was made by experienced geneticists.

6. Statistical analysis

A χ^2 test was used to evaluate the correlation between categorical variables. In survival analysis, Kaplan-Meier survival curves were estimated. A p-value <0.05 was considered statistically significant for all analyses, and all analyses were two-tailed. All data were analyzed using the R software (version 4.1.2).

RESULTS

1. Patient characteristics

In all patients (n=54) with matched pre- and post-NAC ovarian tumor samples, the median age at diagnosis was 59.6 years (range, 40–80 years). The initial FIGO stage was IIIA in 1.9% of patients (1/54), IIIB in 11.1% (6/54), IIIC in 31.8% (17/54), IVA in 1.9% (1/54), and IVB in 53.7% (29/54). All patients underwent diagnostic tumor biopsy and NAC, followed by interval debulking surgery. For NAC, all patients received platinum-based regimen and none of the patients received PARPi treatment; while for maintenance chemotherapy, all patients received platinum-based regimen and one patient received PARPi treatment (**Table S2**). The median follow-up period was 32.0 months. gBRCA pathogenic mutation was identified in 29.7% of patients (16/54), including *BRCA1* mutation in 56.3% (9/16) and *BRCA2* mutation in 43.8% (7/16). According to the three-tier CRS, 7.4% of patients (4/54) had CRS 1, 72.2% (39/54) had CRS 2, and 20.4% (11/54) had CRS 3. Based on radiologic follow-up, 74.1% of patients (40/54) showed partial response and 25.9% (14/54) showed stable disease after NAC. There was no significant association between CRS and treatment response based on radiographic findings. The clinicopathologic parameters are summarized in **Table 1**. In the paired pre- and post-NAC tissues, 48.1% (26/54) were evaluated with same tissue site. On the other hand, 51.9% (28/54) did not have enough residual tumor cells in the same site of pre- and post-NAC samples; therefore, tumor tissues of different site were evaluated (**Table S3**).

https://doi.org/10.3802/jgo.2023.34.e45

Table 1. Clinicopathologic characteristics of patients with ovarian high-grade serous carcinoma (n=54)

Variables	Values
Age (yr)	59.6±10.3
FIGO stage	
IIIA	1 (1.9)
IIIB	6 (11.1)
IIIC	17 (31.8)
IVA	1 (1.9)
IVB	29 (53.7)
gBRCA mutation status	
BRCA1	9 (16.7)
BRCA2	7 (13.0)
VUS	2 (3.7)
Negative	36 (66.7)
CRS	
1	4 (7.4)
2	39 (72.2)
3	11 (20.4)
Neoadjuvant chemotherapy response*	
Partial response	40 (74.1)
Stable disease	14 (25.9)

Values are presented as number (%).
CRS, chemotherapy response score; FIGO, International Federation of Gynecology and Obstetrics; gBRCA, germline BRCA1/2; VUS, variant of unknown significance.
*Based on radiologic finding.

In survival analysis, gBRCA-wildtype cases showed a tendency toward worse PFS and overall survival (OS) compared to gBRCA-mutant cases (p=0.070 and p=0.062, respectively) (**Fig. S1A–B**). Furthermore, CRS 3 cases showed significantly better PFS compared to CRS 1–2 cases, while CRS status was not significantly associated with OS (p=0.048 and p=0.450, respectively) (**Fig. S1C–D**). There were no significant differences in survival according to patient's age, FIGO stage, and therapeutic response based on radiologic finding (data not shown).

2. RAD51 expression before NAC

In the 54 patients with pre-NAC tissues, conventional IHC for RAD51/geminin/γH2AX was performed. After excluding three cases (5.5%) due to low tumor cellularity (<300 viable tumor cells), 12 cases (23.5%) due to less than 25% of γH2AX positive tumor cells, and three cases (7.7%) due to less than 40 geminin-positive tumor cells per high power field, 44.4% (16/36) of RAD51-high and 55.6% (20/36) of RAD51-low cases were identified (**Fig. 1A**). The RAD51 status was not significantly associated with age, initial FIGO stage, gBRCA status, and CRS (**Table S4**). In survival analysis, RAD51-high cases showed significantly worse PFS (p=0.032), while there was no significant difference in OS according to the RAD51 status (p=0.790) (**Fig. 2A and B**). RAD51 status was not significantly correlated with disease progression at either 6 months or 12 months (**Table S4**).

3. RAD51 expression after NAC

Of the 54 patients with post-NAC tissues, all cases showed enough tumor cellularity (≥300 viable tumor cells) and at least 25% of γH2AX expression. After excluding four cases (7.4%) due to less than 40 geminin-positive tumor cells per high power field, 36.0% (18/50) of RAD51-high and 64.0% (32/50) of RAD51-low cases were identified (**Fig. 1B**). RAD51 status was not associated with age, initial FIGO stage, gBRCA status, and CRS (**Table 2**). In the survival analysis of post-NAC tissues, the RAD51-high group showed significantly worse PFS (p=0.013) and tended to show worse OS (p=0.067) (**Fig. 2C and D**). Furthermore, RAD51-high

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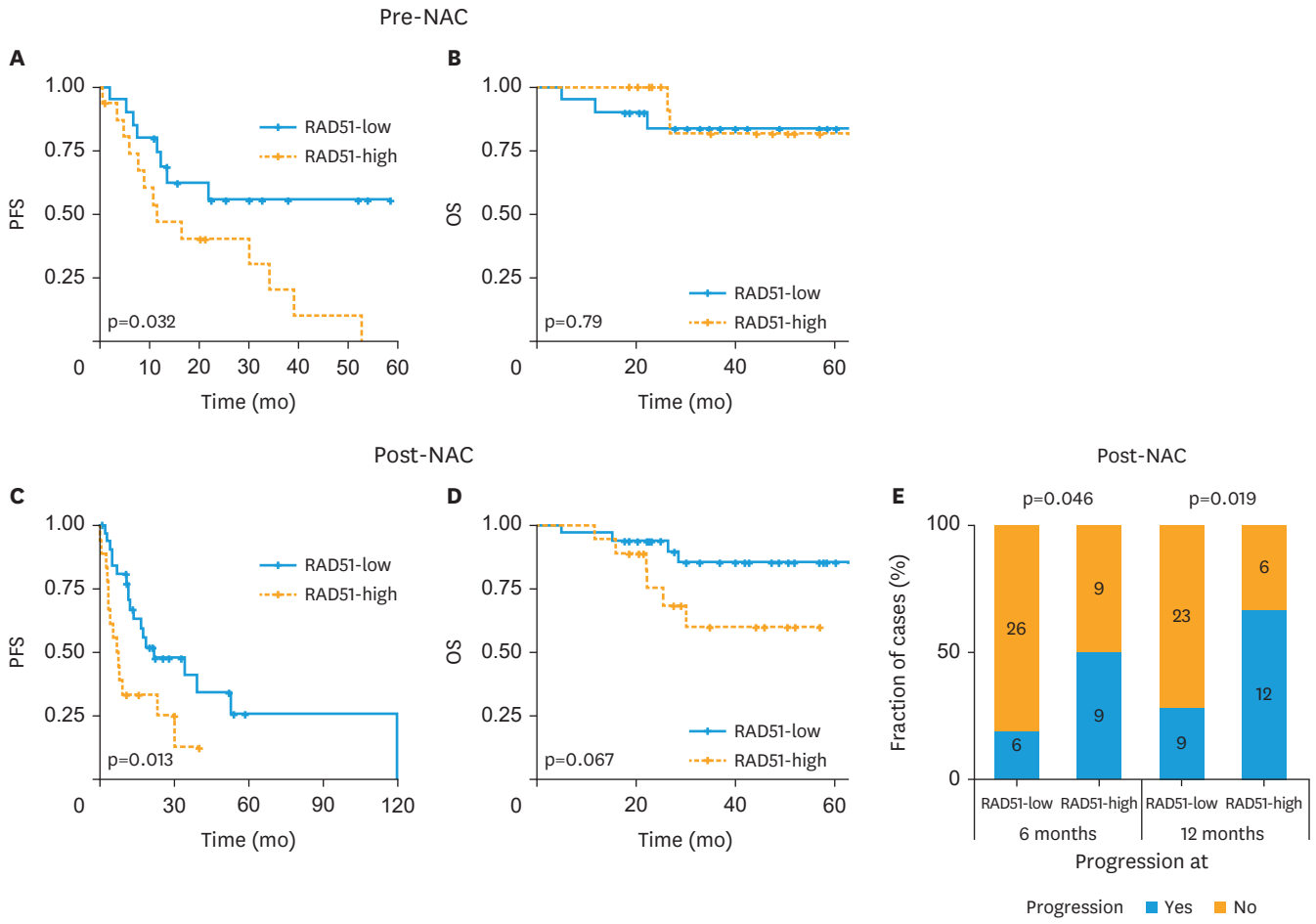

Fig. 2. Survival analysis and prediction of progression according to RAD51 status. (A, B) Survival analysis according to RAD51 status before neoadjuvant chemotherapy in ovarian high-grade serous carcinoma. (C-E) Survival analysis (C, D) and prediction of progression at 6 months and 12 months (E) according to RAD51 status after neoadjuvant chemotherapy in ovarian high-grade serous carcinoma.
NAC, neoadjuvant chemotherapy; OS, overall survival; PFS, progression-free survival.

cases showed a higher risk of progression compared to RAD51-low cases at both 6 months and 12 months (p=0.046 and p=0.019, respectively) (**Table 2** and **Fig. 2E**).

4. Change in RAD51 status before and after NAC

Overall, 34 patients had matched pre- and post-NAC RAD51 results. Of the pre-NAC tissues, 55.9% (19/34) were RAD51-low, and 44.1% (15/34) were RAD51-high cases. Overall, 44% (15/34) of the pre-NAC RAD51 results changed in the post-NAC tissue. In the RAD51-low group before NAC, 73.7% (14/19) of the cases maintained RAD51-low, while 26.3% (5/19) changed to RAD51-high after NAC (**Fig. 3A**). In the RAD51-high group before NAC, 66.7% (10/15) of the cases changed to RAD51-low, and 33.3% (5/15) remained RAD51-high after NAC. To assess the association between the change in RAD51 status and clinical outcomes, the patients were divided into four subgroups: low-to-low (41.1%, 14/34), low-to-high (14.7%, 5/34), high-to-low (29.4%, 10/34), and high-to-high (14.7%, 5/34) groups. In survival analysis, the high-to-high RAD51 group showed the worst PFS and the low-to-low group showed the best PFS (p=0.003), while the four subgroups showed no significant difference in OS (p=0.070) (**Fig. 3B and C**). The change in RAD51 status was not significantly correlated with disease progression at either 6 months or 12 months (**Table S5**).

https://doi.org/10.3802/jgo.2023.34.e45

Table 2. Clinicopathologic characteristics according to the RAD51 status after neoadjuvant chemotherapy in ovarian high-grade serous carcinoma

Variables	RAD51-low (n=32)	RAD51-high (n=18)	p-value
Age (yr)			0.167
<60	22 (68.8)	8 (44.4)	
≥60	10 (31.2)	10 (55.6)	
FIGO stage			0.173
IIIA	0 (0.0)	1 (5.6)	
IIIB	2 (6.2)	3 (16.7)	
IIIC	9 (28.1)	8 (44.4)	
IVA	1 (3.1)	0 (0.0)	
IVB	20 (62.5)	6 (33.3)	
gBRCA mutation status			1.000
Mutant	11 (34.4)	6 (33.3)	
Wildtype	21 (65.6)	12 (66.7)	
CRS			0.956
1	2 (6.2)	1 (5.6)	
2	24 (75.0)	13 (72.2)	
3	6 (18.8)	4 (22.2)	
Progression at 6 mo			0.046
No	26 (81.2)	9 (50.0)	
Yes	6 (18.8)	9 (50.0)	
Progression at 12 mo			0.019
No	23 (71.9)	6 (33.3)	
Yes	9 (28.1)	12 (66.7)	

CRS, chemotherapy response score; FIGO, International Federation of Gynecology and Obstetrics; gBRCA, germline *BRCA1/2*.

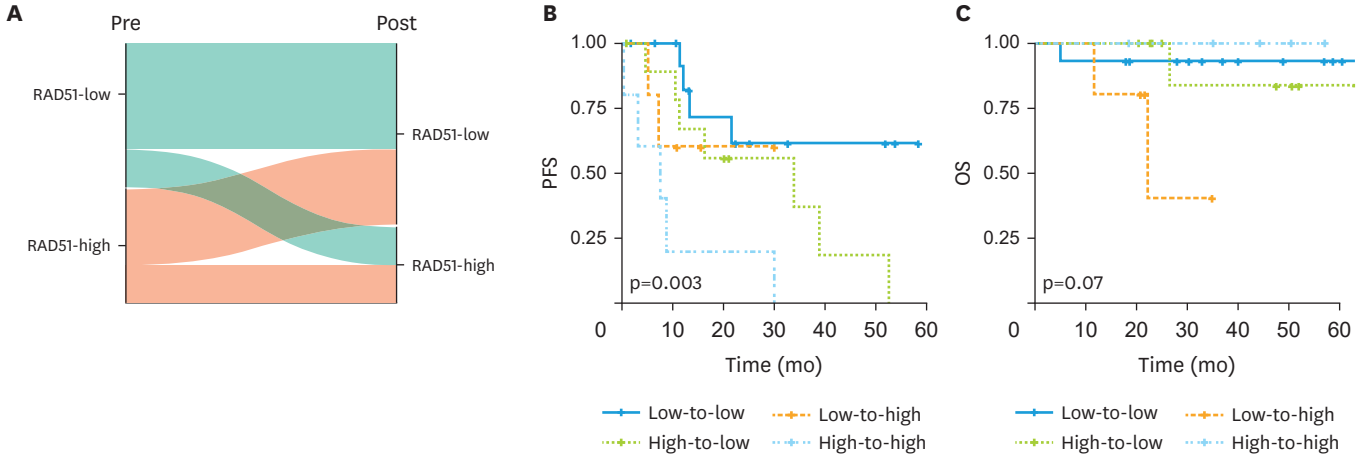

Fig. 3. Changes in RAD51 status before and after neoadjuvant chemotherapy in ovarian high-grade serous carcinoma. (A) Schematic diagram according to change in RAD51 status. (B, C) Survival analysis according to changes of RAD51 status before and after neoadjuvant chemotherapy in ovarian high-grade serous carcinoma. OS, overall survival; PFS, progression-free survival.

5. Fluorescent multiplex IHC in pre-NAC tissues

Fluorescent multiplex IHC was performed in 10 cases of the pre-NAC surgical tissues for the comparison between RAD51 foci evaluation and our method. The cases included five RAD51-low and five RAD51-high cases, according to the categorization by conventional IHC analysis. In RAD51-geminin multiplex IHC, the average percentage of geminin-positive cells with five or more RAD51 nuclear foci among geminin-positive cells were 4% for the RAD51-low group and 58% for the RAD51-high group (**Fig. 4A and B**). Using the criteria for the RECAP test, which regarded <20% RAD51 foci-harboring geminin-positive cells among geminin-positive cells as HRD and ≥50% geminin-positive cells with RAD51 foci as HR proficient (HRP), all RAD51-low cases were classified as HRD and all RAD51-high cases were classified as HRP [17].

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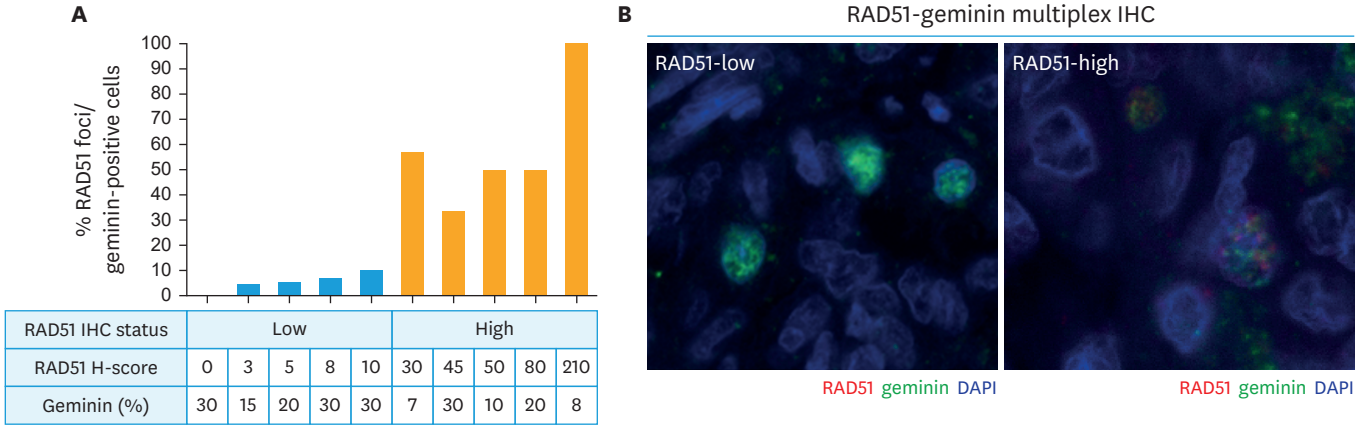

RAD51 IHC status	Low					High				
RAD51 H-score	0	3	5	8	10	30	45	50	80	210
Geminin (%)	30	15	20	30	30	7	30	10	20	8

Fig. 4. Comparison of the RAD51 evaluation by fluorescent multiplex IHC and conventional IHC, and the representative images of multiplex IHC for RAD51-low and RAD51-high ovarian high-grade serous carcinoma. (A) Comparison of RAD51 foci evaluation (RAD51 foci-harboring geminin-positive cells/geminin-positive cells) by fluorescent multiplex IHC and RAD51 status (H-score) and geminin (%) by conventional IHC. (B) The representative images of RAD51-geminin multiplex IHC for RAD51-low and RAD51-high cases.
IHC, immunohistochemistry.

DISCUSSION

HRD tumors may regain HR functionality through genetic reversion, especially after chemotherapy [28,29]. While current genome-based approaches provide a snapshot of past genomic events and cannot reflect dynamic changes in HRD status, the RAD51 assay reflects real-time HRD status, which is regarded as a possible functional HRD assay. Regarding RAD51 measurement methods, IHC is a convenient and cost-effective tool that is desirable for clinical diagnostic practice; however, only a few studies have reported RAD51 IHC evaluation in HGSC [18-20]. To identify the applicability and predictive value of RAD51 toward platinum-based NAC response, we performed RAD51/geminin/γH2AX IHC, which were selected regarding the mechanism of RAD51 pathway, in ovarian HGSC patients with pre- and post- NAC samples.

Different from RAD51 foci evaluation by RAD51 IF assays, we evaluated nuclear RAD51 expression similar to previous studies, as RAD51 nuclear foci are not distinct in conventional IHC [14,16-19]. In addition, due to the lack of standardized RAD51 expression cutoff criteria, we applied H-score 20 as an optimal cutoff based on the maximally selected rank statistics in our study cohort. To identify the concordance with RAD51 foci evaluation, we performed RAD51 foci evaluation using fluorescent multiplex IHC in 10 representative cases. Although the number of the studied cases were limited, RAD51-low/high categorization by conventional IHC was perfectly concordant with HRD/HRP group by RAD51 foci evaluation, suggesting the reliability of our IHC evaluation.

In our RAD51/geminin/γH2AX analysis, high RAD51 IHC expression was significantly associated with worse PFS in pre- and post-NAC HGSC tissues, which further validated previous results [18-20]. Moreover, post-NAC RAD51 status showed a higher association with worse PFS and OS compared to pre-NAC51 status, and only post-NAC RAD51 status could predict progression at both 6 months and 12 months. This might be because the RAD51 status of post-NAC tissue reflects a more recent HRD status than the results of pre-NAC tissue. As the predictive value of RAD51 was higher than the current clinical predictive biomarkers–gBRCA, CRS, and FIGO stage, we suggest the RAD51 IHC assay as a new predictive marker for platinum-based chemotherapy response in ovarian HGSC. For RAD51 evaluation, timing for

https://doi.org/10.3802/jgo.2023.34.e45

the evaluation is very important since HR repair proteins interact at specific time points after DNA damage [13]. Previous studies applied various periods to fixation from DNA damage, but most of them showed correlation between RAD51 and treatment response [13,15-20]. Since post-NAC RAD51 status showed greater predictive power compared to pre-NAC RAD51 status and was accompanied by definite exogenous DNA damage, the time after NAC using interval debulking surgery specimen may be suitable for assessing RAD51 status.

In cases with matched pre- and post-NAC RAD51 results, 44.1% of the cases showed a RAD51 status change, including RAD51 low-to-high and high-to-low cases. In RAD51 low-to-high cases, RAD51-low tumor cells, which are susceptible to platinum chemotherapy, may selectively disappear. In contrast, a significant proportion of RAD51 high-to-low cases were unexpectedly observed. In addition, patients with consistently high RAD51 status showed the worst PFS while consistently low RAD51 group showed the best PFS. These results suggest that RAD51 status could change dynamically, and sequential follow-up of RAD51 status might reflect the biological behavior of HGSCs. As our cohort included a limited number of cases with sequential RAD51 status, further studies using a larger cohort are needed to elucidate the clinical significance of sequential RAD51 evaluation.

BRCA1/2 plays a major role in promoting RAD51 recruitment, and *BRCA1/2* mutation is significantly associated genomic HRD [11,30,31]. However, in this study, RAD51 status was not correlated with gBRCA status, and there are two possible explanations for the discordance. First, since we evaluated gBRCA and not somatic *BRCA*, the tumor might have additional somatic *BRCA* mutations. Second, *BRCA*-mutant tumors may regain HR functionality through genetic reversion, especially after chemotherapy, but also without chemotherapy [28,29]. Therefore, the genomic scar has a possibility of not reflecting the current HR status, while RAD51 assay is a functional assay to show whether the HR pathway currently works in the tumor cells. Similar to our study, Hoppe *et al*. also reported no association between *BRCA* mutation and RAD51 status, suggesting that the mechanisms driving RAD51 expression in cancer are unrelated to the presence of a genomic HRD [24].

In this study, γH2AX positivity was 74.5% and 100% in pre-NAC and post-NAC tissues, respectively. γH2AX is a marker for DNA damage, and previous studies used γH2AX to confirm DNA damage after exogenous DNA damage induction by irradiation, platinum, or PARPi [32-34]. Thus, high γH2AX positivity had been expected in post-NAC tissue, and the difference in γH2AX expression between pre- and post-NAC tissues might have derived from NAC treatment. Meanwhile, significant γH2AX expression in pre-NAC tissues suggested that a substantial number of treatment-naïve ovarian HGSCs harbored endogenous DNA damage, which enabled RAD51 evaluation without exogenous DNA damage.

The strengths of this study were as follows: 1) This was the first study to evaluate RAD51 IHC expression using RAD51/geminin/γH2AX in ovarian HGSC. 2) We identified different predictive potential to platinum-based chemotherapy response in RAD51 expression of paired pre- and post-NAC tissues. 3) We compared the results of RAD51 nuclear expression by conventional IHC with foci evaluation by fluorescent multiplex IHC. The limitations of our study were as follows: 1) A small number of paired samples were evaluated. 2) RAD51 status was not compared with genetic HRD status. 3) RAD51-evaluated paired tissues were not matched in a considerable number of cases due to the limited sampling of pre-NAC tissues and tumor regression of post-NAC tissues. 4) Although PARPi is also associated with HRD, we focused on predicting platinum-based chemotherapy response because platinum-based regimens are used for NAC.

https://doi.org/10.3802/jgo.2023.34.e45

In conclusion, we identified that high RAD51 IHC expression was significantly associated with worse PFS in both pre- and post-NAC of ovarian HGSCs, and post-NAC RAD51 status showed greater predictive power compared to pre-NAC RAD51 status. Moreover, RAD51 status was evaluable in a significant proportion of treatment-naïve HGSC tissue samples. As RAD51 status dynamically changes, sequential follow-up of RAD51 status might reflect the biological behavior of HGSCs.

SUPPLEMENTARY MATERIALS

Table S1

Change in RAD51 status of pre-and post-neoadjuvant therapy specimens in ovarian high-grade serous carcinoma

Click here to view

Table S2

Neoadjuvant and maintenance chemotherapy regimens in ovarian high-grade serous carcinoma (n=54)

Click here to view

Table S3

The analyzed tissue site in pre- and post-neoadjuvant therapy specimens in ovarian high-grade serous carcinoma (n=54)

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Table S4

Clinicopathologic characteristics according to the RAD51 status before neoadjuvant chemotherapy in ovarian high-grade serous carcinoma

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Table S5

Change in RAD51 status of pre- and post-neoadjuvant therapy specimens in ovarian high-grade serous carcinoma

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Fig. S1

Survival analysis according to germline BRCA mutation status and chemotherapy response score. (A, B) Survival analysis of patients with ovarian cancer according to gBRCA mutation status. gBRCA-wild cases showed a tendency toward worse overall survival or progression-free survival. (C, D) Survival analysis of patients with ovarian cancer according to CRS. CRS 3 cases showed significantly worse progression-free survival.

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https://doi.org/10.3802/jgo.2023.34.e45

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