RAD51 as a Biomarker for Homologous Recombination Deficiency in High-Grade Serous Ovarian Carcinoma: Robustness and Interobserver Variability of the RAD51 test

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Supplementary Materials and Methods

Supplementary methods regarding the RAD51 scoring protocol.

Scoring methodology

For this study, a predefined uniform RAD51 scoring methodology was agreed upon. RAD51 scoring was independently performed by a local observer, using their local immunofluorescence microscope. All observers had experience with RAD51 scoring. The following scoring methodology was applied. First, the 20x and 63x objectives were used to review three immunofluorescent markers (DAPI for DAPI, FITC for geminin, and Texas Red for RAD51) to evaluate the tumor tissue, location of (geminin-positive) tumor cells, and staining quality. Based on this initial overall impression of RAD51 foci, the observer determined whether the presence and quantity of RAD51 nuclear foci were proportional ('homogenous') or disproportional ('heterogeneous') distributed among different tumor fields. If the latter was the case, it was differentiated whether this was due to technical issues or artifacts (including necrosis of tumor areas or poorly fixated areas) or whether the tumor was 'RAD51 heterogeneous' (defined as distinct geographical area(s) of geminin-positive cells with RAD51 foci (HR-proficient areas) and GMN+ cells that lack RAD51 foci (HRD areas)). Next, we used DAPI to orientate and select vital tumor areas. At least 100 geminin-positive cells were randomly selected (63x objective) and scored in three to four distinct tumor areas. For selected geminin-positive cells, the number of RAD51 foci per nucleus was determined (0, 1, 2, 3, 4, ≥5) and registered on the RAD51 scoring form (Table S1). Any relevant issues regarding tissue and/or staining quality were noted on the scoring form.

Testing phase

In the testing phase, the interobserver variability was re-determined in an independent set of HGSOC resection and biopsy specimens with known BRCA1/2 PV (n=10; case ID A – J). An updated RAD51 scoring methodology was applied (Table S2), based on the discussions in the evaluation meeting. All cases of the testing cohort were consecutively scored for γH2AX, RAD51, and BRCA1 (Table S2). If the γH2AX, RAD51, or BRCA1 showed obvious intratumoral heterogeneity, each area was separately scored and a rough estimate of the area of each clone was provided. Additionally, clear criteria for 'non-evaluability' were predefined, including, but not restricted to, limited tumor cell percentage and low γH2AX (Table S2).

Table S1. Predefined RAD51 scoring form used in the training phase.

Case ID:	
Date:	
Staining protocol:	o IGR o LUMC o Parma o VHIO
Observer:	
Microscope:	
RAD51 staining:	o Homogenous RAD51 staining o Heterogenous RAD51 staining o Due to artefact/poor fixation o Heterogeneous RAD51 staining
RAD51-/GMN+ cell	
RAD51+/GMN+ cell (1 foci)	
RAD51+/GMN+ cell (2 foci)	
RAD51+/GMN+ cell (3 foci)	
RAD51+/GMN+ cell (4 foci)	
RAD51+/GMN+ cell (≥5 foci)	

Notes:

Table S2. RAD51 scoring form used in the testing phase. New features defined during the evaluation meeting were incorporated into the updated scoring form.

Case ID:……
Staining date:……
Scoring date:……..
Observer:……..
Microscope:……..

1. H&E review

Tissue vitality: o Poor o Moderate o Good
Tumor cell percentage: o < 5% o 5 – 20% o 20 – 49% o ≥ 50%

2. γH2AX/geminin co-IF

Staining pattern: o Homogenous staining o Heterogeneous staining
o Due to artefact/poor fixation
o Heterogeneous γH2AX staining

γH2AX 0 foci:……
γH2AX 1 foci:……
γH2AX 2 foci:……
γH2AX 3 foci:……
γH2AX 4 foci:……
γH2AX ≥5 foci:……

Notes:

3. RAD51/geminin co-IF

Staining pattern: o Homogenous staining o Heterogeneous staining
o Due to artefact/poor fixation
o Heterogeneous RAD51 staining*

RAD51 0 foci:……
RAD51 1 foci:……
RAD51 2 foci:……
RAD51 3 foci:……
RAD51 4 foci:……
RAD51 ≥5 foci:……

* In the case of a heterogeneous RAD51 staining: score each clone (on separate scoring form) and provide area (+, ++, +++).

Notes:

4. BRCA1/geminin co-IF

Staining pattern: o Homogenous staining o Heterogeneous staining
o Due to artefact/poor fixation
o Heterogeneous BRCA1 staining

BRCA1 0 foci:.....
BRCA1 1 foci:.....
BRCA1 2 foci:.....
BRCA1 3 foci:.....
BRCA1 4 foci:.....
BRCA1 ≥5 foci:.....

Notes:

5. Evaluable

o Yes
o No*

* Criteria for non-evaluable cases:

1. H&E
    a. Limited tumor cell percentage (<5%)
    b. Low tumor vitality
2. γH2AX
    a. < 40 evaluable GMN+ cells
    b. Low γH2AX (2 foci and ≤ 25%)
3. RAD51
    a. Quality of geminin staining (including weak geminin)
    b. Brightness of RAD51 staining
    c. Foci-like background in the whole tumor tissue sample