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Tumor infiltrating lymphocytes and homologous recombination deficiency are independently associated with improved survival in ovarian carcinoma

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Abstract

Objective: The presence of tumor infiltrating lymphocytes (TIL) and defects in homologous recombination (HR) are each important prognostic factors in ovarian carcinoma (OC). We characterized the association between HR deficiency (HRD) and the presence of TILs in a cohort of OC patients and the relative contribution to overall survival.

Methods: Patients with carcinoma of the ovary, fallopian tube, or peritoneum were prospectively enrolled. Malignant neoplasm and serum samples were collected. Immunohistochemistry for CD3+ T cells and CD68+ tumor associated macrophages (TAMs) was performed on specimens collected at primary surgery. Damaging germline and somatic mutations in genes in the HR-mediated repair (HRR) pathway were identified using BROCA sequencing. HRD was defined as a damaging mutation in one of 12 genes in the HRR pathway or promoter hypermethylation in *BRCA1* or *RAD51C*.

Results: Ninety-eight of 250 patients included in the analysis had HRD OC (39.2%). HRD OC were enriched for CD3+ TILs and CD68+ TAMs. High CD3+ TIL was present in 65.3% of HRD OC compared to 43.4% of non-HRD OC (p=0.001). High CD68+ TAM was present in 66.3% of HRD OC compared to 50.7% of non-HRD OC (p=0.015). Patients with HRD OC and high CD3+ TILs had the longest median overall survival compared to non-HRD OC with low CD3+ TILs (70.9 vs. 35.8 months, adjusted HR 0.38, 95% CI (0.25-0.59)).

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Author Contribution section

CM, MT, ES, JL, and MK designed and implemented the research. CM, MT, MK, and RG performed histologic analyses. KA, SB, BN, KP, and ES processed and performed genomic assessment. CM and MT performed data analysis. CM and ES drafted the manuscript. All authors reviewed, discussed, and edited the final version of the manuscript.

Conflict of Interest Statement

The authors declare no potential conflicts of interest. All authors have approved the final article.

Conclusions: Patients that have both CD3+ TILs and HRD OC are afforded the greatest improvement in overall survival. This finding may have therapeutic implications for OC patients treated with emerging immunotherapies.

Introduction

Ovarian carcinoma (OC) is the most lethal gynecologic malignant neoplasm with more than 14,000 deaths estimated to occur in the US annually (1). Identifying OC patients most likely to benefit from emerging therapies, especially those targeting the immune system, is critical. Multiple studies have demonstrated an association between the presence of tumor infiltrating lymphocytes (TIL) and survival in patients with OC (2–6). The prognostic implication of having either CD3+ or CD8+ TILs has been further demonstrated in a recent meta-analysis of over 1800 patients from 10 different studies (7). Some investigators have found tumor associated macrophages (TAMs) to be associated with more advanced stages of OC and worse survival, although this association has been inconsistent across studies (8–12).

Similarly, deleterious germline and somatic mutations of some genes in the homologous recombination mediated repair (HRR) pathway, present in more than 20% of OC, also confer a favorable prognosis (13,14). These proteins function to repair double stranded DNA breaks with high fidelity, and mutations in these genes are associated with an increased sensitivity to platinum chemotherapy and improvements in overall survival (13,15–18). Homologous recombination deficiency (HRD) may be conferred by a deleterious mutation in a key gene in the HRR pathway (such as *BRCA1, BRCA2, RAD51C, RAD51D, BARD1, BRIP1*, and *PALB2*), or by promoter hypermethylation of *BRCA1* or *RADS1C*, which results in decreased RNA and protein expression (19–23).

OC associated with germline or somatic mutation in key genes in the HRR pathway may have differences in the tumor microenvironment including immune cell infiltration (24–27). These studies are limited by the small number of included subjects with HRD and a primary focus on *BRCA1* and *BRCA2* (*BRCA1/2)* mutations. In addition, they did not include other potential sources of HRD, such as mutations in other genes in the HRR pathway and promoter hypermethylation of *BRCA1* and *RAD51C* (13,28,29). Nevertheless, these data raise the question of whether the favorable prognosis in HRD OC is secondary to the deficiency in HRR and/or to an improved microenvironment and immune response.

The goal of this study was to clarify the association between HRD and the presence of TILs and TAMs in a large cohort of OC patients and to determine the relative contribution of each on overall survival. We hypothesized that the improved survival observed among patients with HRD OC is conferred, in part, by the presence of higher immune cell infiltration.

Methods

Study subjects

Patients with ovarian, fallopian tube, and primary peritoneal carcinoma (collectively termed OC) who underwent primary surgery at the University of Washington were prospectively enrolled in a gynecologic oncology tumor bank between 1996 and 2011. All subjects

provided informed consent for tissue banking and genetic studies. Subjects who received neoadjuvant chemotherapy were excluded. At primary debulking surgery, fresh tissue was obtained and flash frozen including primary carcinoma, metastases, and paired normal tissue. In cases where the primary site was unknown, such as primary peritoneal cancer, the primary site was arbitrarily defined as the site of the largest intraperitoneal deposit (usually omentum). Optimal surgical cytoreduction was defined as less than 1 cm of residual disease at primary surgery. Subjects were prospectively followed at the University of Washington or through correspondence with the treating oncologist.

Homologous Recombination status determination

Damaging germline and somatic mutations in genes in the HRR pathway were identified using BROCA sequencing as previously described (13,28). HRD was defined as a damaging mutation in one of 12 HRR genes (*ATM, ATR, BARD1, BRCA1, BRCA2, BRIP1, CDK12, NBN, PALB2, RAD51C, RAD51D, XRCC2*) or promoter methylation of *BRCA1* or *RAD51C* (22,29).

Immunohistochemistry and scoring

Formalin-fixed, paraffin-embedded (FFPE) specimens from the primary site were cut and stained for CD3+ T cells to assess for TILs, and stained for CD68, a marker of histiocytes/macrophages. After deparaffinization and rehydration, endogenous peroxidases were blocked and antigen retrieval was performed in citrate buffer (pH 6) and incubated with the primary antibody overnight at 4°C. Primary antibodies were polyclonal rabbit anti-CD3 (Dako/Agilent #A0452) diluted 1:800, and mouse monoclonal anti-CD68 (Dako/Agilent #M0876) diluted 1:200. Slides were then counterstained, dehydrated, and mounted.

All slides were independently scored by a gynecologic pathologist and a pathology-trained gynecologic oncology fellow, both blinded to case designation. For CD3 evaluation of TILs, three high-staining regions in the tumor were identified and scored: 0 (no cells), 1+ (≤5 cells), 2+ (6-19 cells), or 3+ (≥20 cells) per 400x high-powered field (HPF) (12). For CD68 staining of TAMs, three high-staining regions in the tumor were scored: 0 (no cells), 1+ (≤20 cells), 2+ (20-49 cells), 3+ (≥50 cells) per HPF (Figure 1). TILs and TAMs in the stroma were excluded. All samples with a discrepant score were reviewed together at a multi-headed scope to determine a final score. For both CD3 and CD68, fewer than 10% of cases had discrepant scores. Interrater reliability scores were $\kappa = 0.89$ and 0.85 for CD3 and CD68, respectively, indicating very good interrater reliability.

Statistical analyses

Baseline patient characteristics were compared between cohorts based on HRD status using Student's t-test and chi-square tests, where appropriate. Survival was defined as the time from diagnosis until death and patients were censored at the date of last known follow-up. Survival analysis was performed according to the methods of Kaplan-Meier and both unadjusted and multivariable-adjusted analyses were conducted. Eighteen subjects with FIGO stage I carcinoma were excluded from survival analyses. Statistical analyses were performed using STATA (v14.0, College Station, TX).

Results

Two hundred and fifty subjects with FFPE tissues from the primary site were available for TIL evaluation and included in the present study. The HRD assessment for these OC including methylation and mutation data have been previously reported (29). Ninety-eight subjects had HRD OC (39.2%): 44 (17.6%) carcinomas had a *BRCA1* mutation, 17 (6.8%) had a *BRCA2* mutation, 13 (5.2%) had a deleterious mutation in a different HRR gene, and 24 (9.6%) had *BRCA1/RAD51C* promoter methylation. Baseline demographics and patient characteristics were similar between patients with HRD and non-HRD OC except for age at presentation and initial CA-125 (Table 1). HRD OC occurred in younger patients ($P<0.001$) and were associated with a higher serum CA-125 at presentation ($P=0.004$).

The distribution of CD3+ TIL and CD68+ TAM scores for the entire population was assessed and IHC scores were classified as either low (0 to 1+) or high (2+ to 3+) staining (Table 2 and supplementary table 1). Among all patients with OC, 52.0% had a high CD3 TIL score and 56.8% had a high CD68 TAM score. Subjects with CD3+ TIL high scores had longer median overall survival compared to patients with CD3+ TIL low scores, 54.8 months vs. 38.9 months, HR 0.62, 95% CI (0.46-0.84). In contrast, there was no survival difference among subjects with high versus low CD68+ TAM scores, 49.6 months vs. 46.2 months, HR 0.97, 95% CI (0.72-1.32) (Figure 2).

Next, the association between CD3+ TIL and CD68+ TAM scores and HRD status was evaluated (Table 2). Both CD3+ TIL and CD68+ TAM scores were significantly higher in HRD OC. A higher proportion of HRD OC scored high for CD3+ TILs and for CD68+ TAMs. Among HRD OC, 65.3% had high CD3+ TIL scores and 66.3% had high CD68+ TAM scores. In comparison, among non-HRD OC, 43.4% had high CD3+ TIL scores and 50.7% had high CD68+ TAM scores. Median overall survival was significantly greater among subjects with HRD OC, 65.4 months vs. 40.6 months, HR 0.56, 95% CI (0.41-0.78) (Figure 2).

Patients were then categorized according to HRD status and high/low IHC score (Table 3). In both unadjusted and multivariable-adjusted survival analysis, patients with HRD OC and high CD3+ TILs had the longest overall survival when compared to patients with non-HRD OC with low CD3+ TILs. Median overall survival was 70.9 months vs. 35.8 months, adjusted HR 0.38, 95% CI (0.25-0.59). Patients with either high CD3+ TIL or HRD OC (but not both) had intermediate survival of 46.0 months and 54.6 months, respectively (Figure 2). This association was not observed when patients were similarly categorized by the presence of CD68+ TAMs.

Discussion

In this cohort of 250 patients with OC characterized for HRD via germline or somatic HRR mutation or promoter methylation of *BRCA1/RAD51C*, subjects with HRD OC had a significantly higher neoplastic infiltration of both TILs and TAMs. To our knowledge, this is the largest cohort of ovarian carcinomas characterized for TILs and TAMs and comprehensively evaluated for HRD by both methylation and mutation of key HRR genes.

Our data are consistent with previous smaller studies (summarized in Table 4), showing that there are more TILs in HRD OC compared to non-HRD OC.

Consistent with other publications, we confirmed the important survival advantage conferred by the presence of T cells within the tumor epithelium for patients with OC (2–6). A 2012 meta-analysis, which included 1815 patients from 10 studies, found that the presence of both CD3+ and CD8+ TILs was associated with a significant improvement in overall survival, pooled HR 2.24, 95% CI (1.71-2.92) (7). This association was recently confirmed by the Ovarian Tumor Tissue Analysis Consortium, which evaluated 5500 OC, showing a dose-response relationship between the quantity of TILs and survival benefit, which was consistent across various histologies, including endometrioid and mucinous subtypes (6).

Our study is the first to characterize the independent contribution to survival of HRD status and immune infiltration. We found that there was a survival benefit to having both CD3+ TILs and HRD carcinoma, with this subgroup having the greatest median overall survival of 70.9 months, nearly double the median overall survival of patients with non-HRD OC lacking TILs (Table 3, Figure 2). Patients with OC with either high TILs or with HRD had intermediate survival. While the improvement in survival associated with *BRCA1/2* mutations is well-characterized, the independent and additive effect of having both CD3+ TILs and HRD in OC is novel (15, 16).

This finding could have important translational relevance to patients with OC. Immune checkpoint blockade (ICB) has been found to be more effective in cancers with high mutational burdens (30–34), but response to ICB has not tested relative to HRD status. We hypothesize that patients with HRD OC but low or absent TILs have cancers with more immunosuppressive microenvironments that will be less susceptible to current immunotherapies. On the other hand, HRD OC with high TILs may be more effectively treated with immunotherapies. These hypotheses will need to be tested in prospective trials and speak to the importance of classifying HRD in all OC trials, not just those that utilize PARP inhibitors.

In addition to more CD3+ TILs, HRD OC had more CD68+ TAMs than non-HRD OC. However, CD68+ TAMs were not associated with overall survival. A recent meta-analysis, which explored the prognostic effect of TAMs in OC in 794 patients, revealed that neither CD68+ TAMs nor CD163+ TAMs were associated with improved overall survival. In contrast, others report that TAMs, in particular M2 macrophages that adopt an immunosuppressive phenotype after exposure to Th2 mediators, are associated with advanced stage and poor prognosis in OC (8,11,35). A higher ratio of M1 macrophages, which suppress cancer progression, to M2 macrophages may be associated with an improved survival (10). The contribution of TAMs to survival in OC and the relationship of specific macrophage subtypes to HRD status requires further study.

The mechanism that leads to increased TILs in HRD OC is not known. Strickland et al. demonstrated that HRD OC have more predicted neoantigens, which were associated with improved survival (27). An increase in predicted neoantigens or tumor mutation burden, which is also increased in *BRCA1/2* mutated ovarian carcinoma (36), may lead to

lymphocyte recruitment to the tumor microenvironment. Another potential explanatory mechanism includes the interplay between HRD and accumulation of damaged free-cytosolic DNA contributing to activation of the cGAS-STING pathway (37). The relative contribution of these mechanisms, as well as other yet described mechanisms, remains to be determined.

With the increased focus on immunotherapy in the treatment of OC, it will be important to understand how patients with HRD OC respond relative to those with non-HRD OC. Initial reports of nivolumab, an anti-PD-1 antibody, in platinum resistant OC demonstrated encouraging clinical efficacy and there are numerous ongoing studies using ICB (38). We now add another consideration to that assessment. Patients with HRD OC can have varying levels of immune cell infiltration, which may also contribute to a more or less favorable response to ICB.

Strengths of this study include comprehensive assessment of both germline and somatic HRR mutation status using BROCA (28) in combination with promoter methylation of *BRCA1* and *RAD51C*, which have been found to correlate with reduced RNA and protein expression (19–22). In addition, we manually performed immune infiltrate IHC scoring consistent with recently proposed pathology guidelines, which is critical for reproducibility in future studies (39). While we did not assess CD8+ TILs, the published literature is consistent regarding the prognostic implications of either CD3+ or CD8+ TILs (7).

In conclusion, we have demonstrated that HRD OC is associated with an increase in CD3+ TILs as well as CD68+ TAMs. Patients that have both CD3+ TILs and HRD OC are afforded the greatest improvement in overall survival. This finding may have therapeutic implications for OC patients treated with emerging immunotherapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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2: TILs in Melanoma, Gastrointestinal Tract Carcinom. Adv Anat Pathol. 2017;24:311–35. [PubMed: 28777143]

Highlights

- Homologous recombination deficient (HRD) ovarian carcinoma (OC) is enriched for CD3+ tumor infiltrating lymphocytes (TIL).
- Patients with both CD3+ TILs and HRD OC have the greatest overall survival, which may have therapeutic implications.
- These findings highlight differences in the tumor microenvironment that could contribute to differential responses.
- This is especially relevant as novel immunotherapies are being tested for the treatment of ovarian carcinoma.

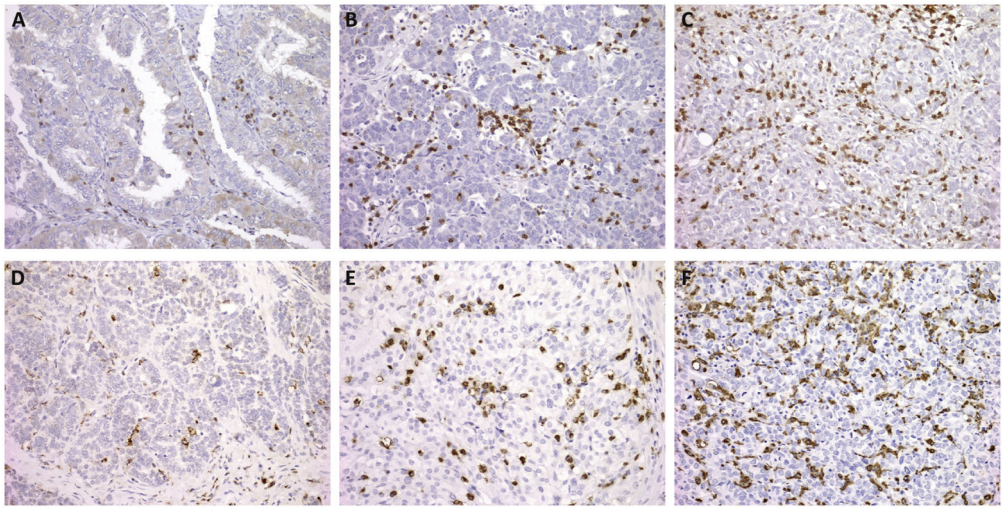

Figure 1.

Representative immunohistochemistry (IHC) staining (200x magnification). IHC staining for CD3+ tumor infiltrating lymphocytes (TILs) are shown for 1+ (A), 2+ (B), and 3+ (C) scores. IHC staining for CD68+ tumor associated macrophages (TAMs) are shown for 1 + (D), 2+ (E), and 3+ (F) scores.

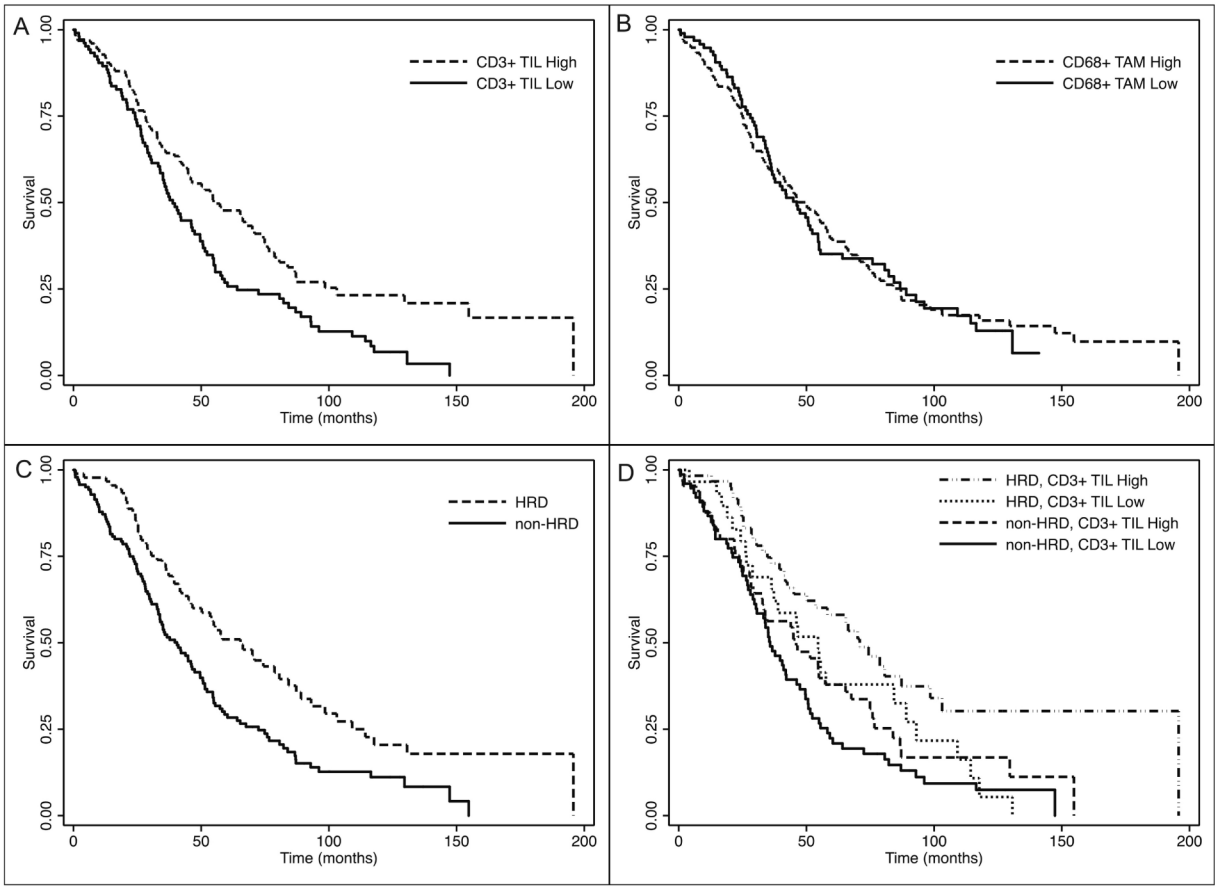

Figure 2.

Kaplan-Meier curves for overall survival by CD3+ tumor infiltrating lymphocyte (TIL) score (high/low) in Panel A, CD68+ tumor associated macrophage (TAM) score (high/low) in Panel B, homologous recombination deficiency (HRD) in Panel C, and by CD3+ TIL score (high/low) and HRD in Panel D.

Table 1.

Baseline demographics by homologous recombination deficiency status.

Characteristic	All (n=250)	non-HRD (n=152)	HRD (n=98)	P-value
Age				
<50	56 (22.4)	23 (15.1)	33 (33.7)	<0.001
50-50	72 (28.8)	40 (26.3)	32 (32.7)	
60-69	62 (24.6)	42 (27.6)	20 (20.4)	
>70	60 (24.0)	47 (30.9)	13 (13.3)	
Disease site				
Ovary	216 (86.4)	131 (86.2)	85 (86.7)	0.090
Fallopian tube	13 (5.2)	5 (3.3)	8 (8.2)	
Primary peritoneal	21 (8.4)	16 (10.5)	5 (5.1)	
FIGO stage				
I	18 (7.2)	11 (7.2)	7 (7.1)	0.381
II	14 (5.6)	10 (6.6)	4 (4.1)	
III	187 (74.8)	115 (75.7)	72 (73.5)	
IV	29 (11.6)	16 (10.5)	13 (13.3)	
Missing	2 (0.8)	0 (0.0)	2 (2.0)	
Histology				
Serous	183 (73.2)	111 (73.0)	72 (73.5)	0.425
Endometrioid	17 (6.8)	10 (6.6)	7 (7.1)	
Clear cell	8 (3.2)	6 (4.0)	2 (2.0)	
Mixed	7 (2.8)	3 (2.0)	4 (4.1)	
Undifferentiated	23 (9.2)	12 (7.9)	11 (11.2)	
Other	12 (4.8)	10 (6.6)	2 (2.0)	
Grade				
1	6 (2.4)	3 (2.0)	3 (3.1)	0.232
2	12 (4.8)	10 (6.6)	2 (2.0)	
3	230 (92.0)	137 (90.1)	93 (94.9)	
Missing	2 (0.8)	2 (1.3)	0 (0.0)	
Optimal cytoreduction				
Yes	167 (66.8)	98 (64.5)	69 (70.4)	0.565
No	81 (32.4)	53 (34.9)	28 (28.6)	
Missing	2 (0.8)	1 (0.7)	1 (1.0)	
Initial CA-125				
0-1000	144 (57.6)	98 (64.5)	46 (46.9)	0.004
1001-2500	50 (20.0)	30 (19.7)	20 (20.4)	
>2500	49 (19.6)	19 (12.5)	30 (306)	
Missing	7 (2.8)	5 (3.3)	2 (2.0)	

Data shown are n (column %). HRD, homologous recombination deficient.

Table 2.

Association of high/low scores for CD3+ TILs and CD68+ TAMs with homologous recombination deficiency.

HRD status	CD3+ TIL score			CD68+ TAM score			
	Low (0-1)	High (2-3)	P value	Low (0-1)	High (2-3)	P value	Total
non-HRD	86 (56.6)	66 (43.4)	0.001	75 (49.3)	77 (50.7)	0.015	152
HRD	34 (34.7)	64 (65.3)		33 (33.7)	65 (66.3)		98
BRCA1 mutation	15 (34.1)	29 (65.9)		17 (38.6)	27 (61.4)		44
BRCA2 mutation	9 (52.9)	8 (47.1)		5 (29.4)	12 (70.6)		17
Other HRR mutation	4 (30.8)	9 (69.2)		4 (30.8)	9 (69.2)		13
BRCA1/RAD51C hypermethylation	6 (25.0)	18 (75.0)		7 (29.2)	17 (70.8)		24
Total	120 (48.0)	130 (52.0)		108 (43.2)	142 (56.8)		250

Data shown are n (row %). P value indicates the comparison of non-HRD to HRD ovarian carcinoma. TIL, tumor infiltrating lymphocytes; TAM, tumor-associated macrophages; HRR, homologous recombination-mediate repair, HRD homologous recombination deficient.

Table 3.

Unadjusted and multivariable-adjusted association of CD3+ TILs or CD68+ TAMs and homologous recombination deficiency with OS.

HRD status	IHC score (high/low)	No.	Median OS (months)	HR (95% CI)	P value	Adjusted HR (95% CI)	P value
non-HRD	CD3 low	76	35.8	1 (Reference)	<0.001	1 (Reference)	<0.001
	CD3 high	65	46.0	0.75 (0.52-1.0)		0.65 (0.44-0.95)	
HRD	CD3 low	30	54.6	0.71 (0.45-1.13)		0.71 (0.44-1.14)	
	CD3 high	61	70.9	0.40 (0.27-0.61)		0.38 (0.25-0.59)	
non-HRD	CD68 low	67	40.6	1 (Reference)	0.004	1 (Reference)	<0.001
	CD68 high	74	35.1	1.04 (0.72-1.51)		0.89 (0.61-1.30)	
HRD	CD68 low	31	84.3	0.55 (0.32-0.93)		0.56 (0.33-0.96)	
	CD68 high	60	65.4	0.59 (0.39-0.90)		0.54 (0.35-0.83)	

Adjusted for age, stage, and optimal surgical cytoreduction. Stage 1 subjects (n=18) excluded. TIL, tumor infiltrating lymphocytes; TAM, tumor-associated macrophages; HRD homologous recombination deficient; OS, overall survival; IHC, immunohistochemistry.

Table 4.

Summary of studies characterizing the tumor microenvironment in homologous recombination deficient ovarian carcinoma.

Author (year)	No. cases	No. HRD (%)	IHC markers	Associations with HRD ovarian carcinoma
Morse (current)	250	98 (39.2)	CD3, CD68	HRD ovarian carcinoma enriched for both CD3+ TILs and CD68+ TAMs, HRD and CD3+ TILs each independently prognostic for improved OS
Clarke (2009) (26)	40	18 (45.0)	CD3, CD4, CD8, CD20, CD43, CD 117, granzyme-B	CD8+ TILs correlated with *BRCA1* mutation or loss of expression and with improved OS
McAlpine (2012) (24)	131	52 (39.7)	CD3, CD4, CD8, CD20, FOXP3, and TIA-1	Higher CD20+ and TIA-1 immune infiltrate in cases with *BRCA1/2* mutations or *BRCA1* methylation
Soslow (2012) (25)	43	31 (72.1)	TIL assessment not specified	TILs enriched in *BRCA1*, but not *BRCA2* mutated ovarian carcinoma
Strickland (2016) (27)	53	37 (69.8)	CD3, CD4, CD8, CD20, PD-1, and PD-L1	CD3+ TILs and CD8+ TILs enriched in HRD ovarian carcinoma and CD3+ TILs associated with improved OS

HRD, homologous recombination deficient; IHC, immunohistochemistry; TIL, tumor infiltrating lymphocytes; TAM, tumor-associated macrophages; OS, overall survival.