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The CD47 “don’t eat me signal” is highly expressed in human ovarian cancer

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Abstract

Objectives—The CD47 “don’t eat me” signal allows tumor immune evasion. We tested the association of CD47 expression with outcomes in EOC.

Methods—CD47 expression was examined within the TCGA database for ovarian carcinoma. For validation, IHC was performed on a TMA consisting of specimens from 265 patients with EOC. The medical records of the patients were also retrospectively reviewed to correlate demographic and survival data.

Results—CD47 was amplified in 15/316 (5%) ovarian serous cancers in TCGA. In the validation cohort, the majority of patients had stage III/IV disease (208/265, 78.4%). CD47 expression was seen in 210/265 (79.2%). Patients were categorized into CD47hi (129/265; 48.7%) versus CD47lo (136/265; 51.3%). Patients with CD47lo tumors were more likely to have a complete response to adjuvant therapy than CD47hi (65% vs 50%, $p=0.026$). Although there was a trend towards an increase in median OS (37.64 vs 45.26 mos, $p=0.92$) in the CD47lo group compared with CD47hi, the difference was not significant.

Conclusions—CD47 is expressed at high frequency in EOC. Patients with CD47lo EOC had a better treatment response to standard therapy, and trended towards improved OS. This demonstrates that while CD47 may be an immunologic shield that may be considered for targeted therapies, it is likely that it operates in concert with other mechanisms of immune evasion. Future studies to evaluate CD47 expression with other known mechanisms of immune escape in the tumor microenvironment may help further define its role.

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CONFLICT OF INTEREST

The authors have no conflicts of interest.

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Keywords

CD47; SIRP α ; ovarian cancer; CD4⁺ T cells; NY-ESO-1; IFN- γ ; CD8⁺ T-cell activation; anti-tumor effect

INTRODUCTION

CD-47 is a transmembrane protein that interacts with several molecules including integrins, thrombospondins, and signal regulatory protein alpha (SIRP α) [1]. It plays a role in cellular proliferation, apoptosis, adhesion, migration and in immune system homeostasis. Upon binding CD47, SIRP α initiates a signaling cascade via recruitment of the src homology-2 domain containing protein tyrosine phosphatases SHP-1 and SHP-2, which in turn deliver inhibitory signals for phagocytosis [2]. In this way, CD47 serves as a “don’t eat me signal” and a marker of self, as loss of CD47 leads to homeostatic phagocytosis of aged or damaged cells [3]. Thus, when CD-47 protein is expressed on the surface of solid tumor cells, it serves as a potent signal to aid escape from immune surveillance [4]. This enables tumor cells to grow and divide uninterrupted by avoiding yet another host immune mechanism. This phenomenon was first seen in the hematologic compartment, in which red blood cells, lymphocytes, and platelets escape splenic macrophage attack by expressing CD-47 on their surface [2, 5].

CD47 is expressed at a higher level in epithelial ovarian cancer cells compared with normal ovarian cells [6]. Weissman *et al.* evaluated expression of CD47 on solid tumors of several histological types, including ovarian, breast, colon, bladder, glioblastoma, hepatocellular carcinoma, and prostate cancer [2]. It was shown by this group that increased CD-47 mRNA expression correlated to poorer survival outcomes in several solid tumors. Furthermore, Lin *et al.* observed that increased CD47 expression is a poor prognostic factor in ovarian cancer [7]. Based on these reports, we propose a model in which ovarian cancer cells utilize CD47 expression to prevent phagocytic elimination by innate immune cells. To test this hypothesis, we examined the expression of CD-47 in “The Cancer Genome Atlas” (TCGA) database, and validated the results in a tissue microarray (TMA) of over 250 ovarian cancer patients.

METHODS

TCGA analysis

We examined CD47 expression in “The Cancer Genome Atlas” (TCGA) database for ovarian carcinoma as previously described [8, 9]. All genomic data and clinical data of ovarian serous cystadenocarcinoma were downloaded from TCGA data cbiportal (n=316). These downloaded data files including four gene-level data types (copy number variants, gene expression, somatic mutation, and DNA methylation) and clinical data were imported into a relational database which was defined based on the downloaded tab delimited files [8, 9].

Patient Population and Tissue Microarray

The cases for the tissue microarray (TMA) were selected from 265 patients who were diagnosed with ovarian, fallopian tube, and primary peritoneal cancer between 1995 and 2007 at Roswell Park Cancer Institute and were identified retrospectively by data base search from the archives of the Roswell Park Ovarian Cancer Database. Formalin-fixed paraffin-embedded (FFPE; for immunohistochemistry) tissue was available from all patients through the Department of Pathology. All of the specimens were reviewed in our institution, with the histopathologic subtype classified via World Health Organization (WHO) guidelines [10]. The main primary exclusion criteria were poor material quality or quantity (initial biopsy material inadequate, insufficient remaining tumor tissue volume or tissue preservation). This TMA included all available epithelial ovarian cancer subtypes, normal “control” tissues were included on the TMA for comparison. We identify these above three cancers collectively as EOC, secondary to the presumed common origin in the mullerian epithelium. Detailed clinical information for each patient was available from the files of the aforementioned database. The medical records of the patients were also retrospectively reviewed under an approved Institutional Review Board protocol. The review included out- and inpatient treatment, including surgery and chemotherapy. Study outcomes included overall survival and time to progression, each measured from the time of definitive surgery. Progression was defined as objective evidence of recurrence because all therapy was given in the adjuvant setting. The duration of overall survival (OS) was the interval between diagnosis and death. Observation time was the interval between diagnosis and last contact (death or last follow-up). Data were censored at the last follow-up for patients with no evidence of recurrence, progression, or death.

IHC Staining for CD47 and interpretation

Immunohistochemical (IHC) analysis was performed using FFPE tissues from 265 patients on tissue microarrays (TMA). TMA were constructed using 0.6 mm FFPE tissue cores punched from each donor block. To overcome tumor heterogeneity, three representative cores were selected from each tumor. 5µm thick TMA sections were deparaffinized and pretreated in citrate buffer pH 6.0 (Dakocytomation, Carpinteria, CA) for 20 minutes using an ordinary vegetable steamer. Slides cooled 20 minutes and were incubated 10 minutes with 3% H₂O₂ to quench endogenous peroxidase activity. Blocking was performed using serum-free protein block, Dakocytomation (Carpinteria, CA) for 20 minutes. Anti-CD47 polyclonal (Atlas, Stockholm Sweden) was added at 1:50 dilution to each section and incubated 60 minutes at room temperature. Rabbit IgG (Dakocytomation, Carpinteria, CA) was incubated as our negative isotype matched control. Labeled streptavidin biotin (LSAB2) reagents Dakocytomation (Carpinteria, CA) were used according to the manufacturer's instructions followed by a 5 minute incubation with 3,3'-diaminobenzidine (DAB)+ Dakocytomation (Carpinteria, CA). Sections were counterstained with hematoxylin and cover slipped.

Immunohistochemical scoring

For assessment of CD47 expression, the stained TMA slides were evaluated at 40× magnification. Each spot was independently assessed in a blinded fashion. The TMA was

scored based on membranous and cytoplasmic staining of CD47 antibody. A scale of 0–3 was used; 0-absent staining, 1-faint membranous positivity, 2-at least 1/3 cells showed membranous and/or cytoplasmic expression, 3-intense membranous and/or cytoplasmic expression of at least 2/3 of the tissue section. For all TMA slides we had multiple positive and negative controls on the same slide. Scoring was performed in a completely blinded fashion, and consensus score was achieved between two reviewers if there was a conflict. Also, CD47 has predominant membranous staining pattern, with some neoplastic cells showing cytoplasmic expression. No nuclear staining is observed. Any staining in lymphatic, endothelial, lymphocytic, or desmoplastic tissue was discounted. Only epithelial staining was taken into account, independent of whether it was a neoplastic or normal tissue. The external controls (kidney, lung, liver, prostate, adrenal, breast, colon, myometrium, stomach, ovary, testes) were used to assure equal and adequate staining across the TMA itself. In the case of more than one stained population the most intensely stained subpopulation defined the score, based on the presumption that the tumor cells with the highest CD47 expression are ultimately relevant for the biological behavior. Each patient sample had three representative tissue samples and were each scored independently, after which time the scores were averaged for each patient.

Biostatistical analysis

After the scoring was complete, and after empirically investigating a range of cutpoints, including tertiles and quartiles, we found the median was most representative and provides the most statistical power with evenly sized groups. Therefore, we split the CD47 values into two evenly sized groups (high [≥ 1.5] and low expression [< 1.5]). Association between CD47 expression and categorical variables was tested by chi-square tests and continuous variables were tested by two-sample t-tests. Survival probabilities were calculated by Kaplan-Meier.

RESULTS

TCGA analysis

Review of the TCGA database for ovarian serous cystadenocarcinoma revealed that 5% of ovarian cancers profiled had a copy number alteration of the CD47 gene (15 patients of 316) [9, 10]. Of these 15 patients with DNA alterations, 11 had 1cm or less residual disease at time of primary debulking surgery, 13 were stage IIIC (1 IIIB and 1 IV), 14 were grade 3, 6 were platinum sensitive, and 8 had recurred or progressed at time of data analysis. In this exploratory analysis, median survival in the cases with alteration in CD47 gene was 58.05 months versus 44.45 months in the cases without gene amplification ($p=0.162$). In all 15 patients TP53 was mutated, 20% had a mutation in BRCA1 and 13.3% in BRCA2. We investigated these alterations further with cBioportal where copy number alterations were associated with an increase in CD47 expression (Supplementary Figure 1). Also, within the dataset, CD47 mRNA expression was altered in 58 of 489 cases (12%) with Agilent microarray data, with 6% up-regulation and 6% downregulation. There was no difference in cases with alterations versus not (OS $p=0.35$, DFS $p=0.343$). Although there were no differences in DFS and OS from the exploratory analysis of TCGA data, we reasoned that because of the biologic importance of the CD47 pathway, further exploration and validation

of CD47 in ovarian cancer was warranted. Moreover, although the survival difference among the cases with DNA copy number alterations did not reach statistical significance, there is likely some clinical significance to the 13 month difference noted in median survival. Therefore, these results prompted us to further evaluate CD47 as a potential prognostic biomarker in ovarian cancer.

Patient Population and Tissue Microarray

A total of 265 tissues from patients with ovarian, primary peritoneal and fallopian tube cancer were analyzed using immunohistochemistry. The characteristics of the patients in this study are presented in Table 1. As expected, the majority of patients presented with late-stage disease (78%). There were 48 patients that were Stage I/II and 208 were Stage III/IV (9 patients were unstaged/unknown) and most patients had serous histology (164/265; 62%), followed by mucinous, endometrioid, clear cell, and non-epithelial at relatively similar rates (Table 1). The correlation of histology with outcomes and CD47 expression was non-significant.

Expression of CD-47 in Ovarian Cancer

Expression of CD-47 was evaluated with immunohistochemistry (Figure 1). CD47 expression was found in all but 55 of the 265 ovarian cancer specimens (79%). Therefore, we classified tissues based on the degree of expression as CD-47 “high” versus CD-47 “low.” The median value for scoring of tumor staining intensity was found to be 1.5 and this value was utilized to determine “high” versus “low.” Using this definition, a high CD47 protein expression was found in 129/265 (48.7%) of patients with ovarian cancer. There were no differences in the frequency of platinum sensitive patients in the “CD-47 High” and “CD-47 Low” groups (53 and 58 patients respectively). Similar findings were seen in platinum resistant patients, with 42 vs 38 patients in the “CD-47 High” and “CD-47 Low” groups respectively. The median ages of the groups were 63.67 and 62.58 for each group respectively. However, there were 10 patients noted with “CD-47 High” status that were platinum refractory, vs only 3 in the “CD 47 low” group, though this was not statistically significant, with a p-value of 0.1339.

Correlation of CD47 expression with clinical outcome

Median overall survival (OS) was 37.64 months in the “CD47 high” group vs 45.26 months in the “CD47 low” group, although this did not reach statistical significance with log-rank p-value of $p=0.92$. Similarly, though statistically not significant with a p value of 0.632, the median progression free survival (PFS) was noted to be 21.86 months in the “CD47 High” group, vs 24.6 months in the “CD47 Low” group.

There were, however, significant differences for those with high versus low CD-47 in regard to response to primary therapy. “CD47 low” is more likely than “CD47 high” to have a complete response to adjuvant chemotherapy (65% vs 50%, t-test $p=0.02636$).

DISCUSSION

In the present study, our aim was to explore the association between CD47 and outcomes of patients with epithelial ovarian cancer. Although CD47 is expressed in some normal tissues [11], it is not expressed in normal ovaries, but becomes aberrantly expressed in ovarian cancer. We examined the TCGA database and also utilized a TMA consisting of specimens from more than 250 patients. While the TCGA analysis indicated no difference in outcomes in cases with up-regulation vs down-regulation, our immunohistochemical analysis of a large TMA showed high CD47 protein expression in 129/265 (48.7%) of patients, and prognosticated poor response to primary therapy in ovarian cancer. Since CD47 signaling also converges on SHP1 and SHP2 phosphatases, signaling molecules that are utilized by the PD1/PD-L1 pathway to disable anti-tumor T cells [12], the CD47's "don't eat me" signal may represent another mechanism of immune escape in human ovarian cancer. With regard to OS and PFS, our IHC analyses are concordant with the TCGA dataset.

In a univariate analysis by Weissman *et al* [6], patients with solid tumors were also categorized as "CD47 high" or "CD47 low," and high CD47 mRNA expression levels were associated with a decreased progression-free and overall survival. These results suggest that CD47 expression levels may be a clinically relevant prognostic factor in many solid tumors. Furthermore, mice given anti-CD47 antibody showed significant tumor shrinkage with little to no unacceptable side effects [6]. The most prevalent adverse event was anemia, secondary to CD-47 being normally expressed by hematopoietic cells. In another study by Weissman *et al.*, therapeutic doses of blocking anti-mouse CD47 mAbs (clone MIAP301) to normal C57BL/6 mice produced no significant toxic effect except isolated neutropenia [13]. Furthermore, *in vivo* studies of the antibody by the Weissman's group was shown to eliminate and/or prevent tumor metastasis to the lymph nodes and lungs.

The strength of our study is the large sample size of ovarian cancer patients with diverse histologies and treatment outcomes. Therefore, the results are likely representative of the true CD47 expression pattern and prognostic implication in ovarian cancer patients. In contrast to the data by Weissman *et al*, our results indicate that although low expression is associated with better treatment response, this did not translate to a significant prolongation of progression-free and overall survival. These results suggest that while CD47 may in fact be an immunologic shield that may be considered for targeted therapies, it is likely that it operates in concert with other mechanisms of immune evasion in ovarian cancer. These mechanisms include the PD-1/PD-L1 and LAG-3 pathways [14, 15] as well as Tregs and MDSCs [16]. Therefore future studies are warranted to further elucidate how CD47 may conspire with other known mechanisms of immune escape in the tumor microenvironment to allow ovarian cancer to escape from immune attack.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

EOC	Epithelial ovarian cancer
TMA	Tissue microarray
TCGA	The Cancer Genome Atlas
IHC	Immunohistochemistry
OS	Overall Survival
SIRPα	Signal Regulatory Protein alpha
FFPE	Formalin-fixed paraffin-embedded
WHO	World Health Organization

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Highlights

- CD47 serves as a “don’t eat me signal.”
- 5% of ovarian cancers profiled had an alteration of the CD47 gene in the TCGA dataset.
- Low CD47 expression correlates with complete response to therapy.

Figure 1. CD-47 IHC staining

Specimens were stained with Anti-CD47 polyclonal antibody. The external controls were used to assure equal and adequate staining across the TMA itself.

Table 1

Patient characteristics by CD47 Expression.

Clinical and Pathologic features	CD47 Low	CD47 High	P value
All EOC (n)	127	129	
Age (years)	62.58	63.67	
PFS	24.6	21.86	0.63
OS	45.26	37.64	0.92
FIGO Stage			0.16
Early stage (I–II)	23	25	
Late Stage (III–IV)	104	104	
Tumor Grade			0.60
1	12	11	
2/3	117	118	
Histology			0.89
Serous	82	82	
Clear Cell	9	11	
Endometrioid	8	6	
Mucinous	8	6	
Non-epithelial	10	6	
Other	14	14	
Platinum Status			0.13
Sensitive	58	53	
Resistant/Refractory	38	42	
Refractory	3	10	
Clinical Response			0.037
Complete	65	55	
Persistent	34	54	