RESEARCH ARTICLE

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Immunohistochemical Expression of *CD47* and *CD68* in Breast Carcinoma and Their Prognostic Value

Sara Alhanafy[1]*, Heba Hany[1], Khaled Nasef[2], Iman El-Salkh[1], Amira K El-Hawary[1]

Abstract

Background: Cluster of differentiation 47 (*CD47*) has been identified as a new immune checkpoint. The exact role of *CD47* in prognosis of breast cancer remains unclear. This study aims to evaluate immunohistochemical (IHC) expression of *CD47* in breast cancer, and to measure the density of tumor associated macrophages (TAMs) infiltration by *CD68* IHC staining. Furthermore, assessing the relations of *CD47* and *CD68* expression to different clinicopathological variables and evaluating the prognostic role of *CD47* and *CD68* in breast cancer cases. **Methods:** This retrospective cohort study included 200 diagnosed primary breast cancer cases who underwent surgical resection at the Oncology Center of Mansoura University (OCMU), Faculty of Medicine, Egypt. Clinicopathological and survival data were collected. IHC for *CD47* and *CD68* was performed. **Results:** Among 200 breast cancer cases, high *CD47* expression was detected in 89 cases (44.5%). *CD47* high expression was significantly associated with presence of distant metastasis (P=0.04), advanced TNM stage (P=0.02), ER & PR negativity (P=0.04 & 0.004 respectively), and molecular subtype (P=0.03). Their was a statistically significant association between *CD47* and *CD68* expression (P=0.002). *CD47* high expression was found to predict poor overall survival, but it is not considered alone as independent poor prognostic factor by multivariate analysis. Multivariate analysis spotted combined high expression of *CD47* and *CD68* as an independent prognostic predictor for shorter OS in breast cancer patients (P=0.002). **Conclusion:** *CD47* high expression is related to poor prognosis in breast cancer patients especially when associated with high *CD68*+TAMs infiltration. Therefore, *CD47* is a promising prognostic and therapeutic target in breast carcinoma that may direct selection of patients for immunotherapy.

Keywords: Tumor associated macrophages- breast cancer- survival- immunotherapy

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Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in women worldwide, with an estimated 2.3 million new cancer cases and 685,000 cancer deaths in 2020 [1]. The development and progress of breast cancer is an extremely complex process, involving multiple factors in tumor cells and the supporting microenvironment that includes inflammatory and immune cells, and connective tissue cells [2]. The recent success of immunotherapies has increased interest in the immune status of breast cancer where macrophages are the most common immune cells [3]. Within the tumor microenvironment (TME), tumor associated macrophages (TAMs) polarize into two phenotypes, M1 and M2. Whereas M1 macrophages function as promotors of inflammation, M2 macrophages are immunosuppressive and promote tumor progression [4]. TAMs are generally characterized by the expression of cell surface marker *CD68* which is considered a pan macrophage marker that recognizes both M1 and M2 macrophages [5].

CD47 has been found to be highly expressed in various types of human cancer, and its expression is closely associated with the differentiation, metastasis, survival, and prognosis of tumors. It binds to the receptor signal-regulatory protein-α (SIRP-α) on the macrophages to inhibit normal phagocytosis [6,7]. *CD47*-mediated therapy to enhance innate immune activity is a new direction for cancer treatment by blocking *CD47*-SIRPα binding between tumor cells and TAMs to increase phagocytosis of tumor cells [8,9]. Furthermore, blocking the *CD47*-SIRPα axis not only motivates macrophage phagocytosis but also drives the CD8+ T cells mediated elimination of immunogenic tumors [10]. Therefore, *CD47* immunotherapy continues to develop their applications in various solid and hematological tumors [11].

In breast carcinoma, the exact role of *CD47* remains unclear. This study aimed to examine the expression of

*[1]Department of Pathology, Faculty of Medicine, Mansoura University, Mansoura, Egypt. [2]Medical Oncology Unit, Internal Medicine Department, Faculty of Medicine, Mansoura University, Mansoura, Egypt. *For Correspondence: saraalhanafy40@gmail.com*

CD47 and *CD68* in breast carcinoma samples and to evaluate their association with different clinicopathological variables and patient's prognosis.

Materials and Methods

This retrospective cohort study was conducted on 200 formalin-fixed, paraffin-embedded (FFPE) tissue blocks of breast cancer cases naive to preoperative chemotherapy or radiotherapy diagnosed at the laboratory of Pathology, Oncology Center of Mansoura University (OCMU), Faculty of Medicine, Mansoura University, Egypt, during the period from January 2016 to December 2018.

The clinicopathological data of the included 200 cases were retrospectively retrieved from the pathologic database of the OCMU, including: patients' age, size of tumor, skin invasion, nodal status, local recurrence, distant metastasis, and tumor stage according to the updated 8th edition of the American Joint Committee on Cancer (AJCC) [12]. The H&E-stained slides were reviewed to assess the histopathological type of tumor, grade of tumor and the presence lympho-vascular emboli. Based on the immunohistochemical expression of *ER, PR, HER2* and *Ki-67*, cases were classified according to the recommendations of the Gallen International Expert Consensus Report (2013) into 5 intrinsic molecular subtypes: luminal A (ER+ and/or PR+, *HER2*− and *Ki-67* <14%); luminal B/*HER2* negative (ER+ and/or PR+, *HER2*− and Ki- 67≥14%); luminal B/*HER2* positive (ER+ and/or PR+, *HER2*+, any Ki- 67); *HER2* enriched (*HER2*+ and ER−/PR−) and triple negative subtype (ER-, PR- and *HER2*-).

Cases were followed up for 5 years. The follow-up data were collected via accessing patients' medical records, and telephone-based patient or relative interviewing. The follow-up data of concern included: the follow-up duration registered in months, the presence or absence of relapse either local recurrence or distant metastases, disease-free survival (DFS) that was considered as the period from the date of primary surgery to the date of a documented relapse, disease-related mortality, and finally the overall survival (OS) that was calculated from the date of primary surgery till the time of disease specific death or last follow up.

Tissue Microarray Construction

The tissue microarray blocks (TMA) were constructed using a completely manual validated technique [13]. Nine TMA blocks were constructed including three representative cores from each case of the studied 200 cases. Multiple cores of normal tissues (pancreas, thyroid, prostate & spleen) were inserted in each block according to a pre-designed map to serve as orientation and navigation markers and as positive control tissues for the stained IHC markers (prostate is the control tissue for *CD47* and spleen is the control tissue for *CD68*).

Immunohistochemistry

IHC was performed with Autostainer Link 48, using its optimized reagents with pharmDx kits EnVisionTM FLEX Visualization Systems (Link code K8000) and EnVision FLEX Hematoxylin (Link code K8008) according to the user's-guide standardized procedure pre-programmed into the autostainer software.

The interpretation of IHC was done semi-quantitatively and independently by two examining pathologists (AS, HH) using an ordinary light microscope, then scoring was done for each antibody based on its most appropriate specific scoring technique/ system. *CD47* staining was localized to the cytoplasm or cell membrane of tumor cells, and *CD68* staining was found in the cytoplasm, and in some cases also on the plasma membrane of TAMs. Anti-*CD47* (Rabbit polyclonal antibody, catalog number A1838, ABclonal, at dilution of 1:100) was used and the immunoreactivity scoring (IRS) system was done using the staining intensity and the percentage of positive cells. The staining intensity was categorized into four grades as follows: 0 standed for no immunostaining (non-staining), 1 standed for weak (light yellow), 2 standed for moderate (brown yellow) and 3 standed for strong (dark brown). The percentage of positive cells was categorized into five grades as follows: 0 standed for none, 1 standed for 1–10%, 2 standed for 11–50%, 3 standed for 51–80% and 4 standed for > 80%. Three most representative fields of high magnification (x400) in each individual case were selected to calculate the final score. Multiplication of the staining intensity and the percentage of positive cells resulted in an IRS ranging from 0 to 12 for each individual case. A case with high expression of protein was scored when scoring between 7 and 12, while a case with low expression of protein was scored when scoring between 0 and 6 [7]. For evaluation of TAMs, anti- *CD68* (Mouse monoclonal antibody, catalog number IR609, Clone KP1, Dako, ready to use) was used and evaluated by counting the number of positive cells with a macrophage morphology by adapting the reported hotspot quantitative method. The triplicate cores of each specimen were screened at low magnification (×100), and five areas with the greatest number of positively stained cells (hot spot area) were selected for further analysis. The mean macrophage count in these areas was estimated for each case at high power magnification (×400). For statistical analyses, the number of positive cells was divided into lower and higher groups based on cut-off points according to the median: low representing less than or equal than the median, and high representing greater than the median [14].

Statistical analysis

Statistical analyses were done using SPSS 25.0 (IBM Corporation, New York, USA). The association between *CD47* and *CD68* expression with clinicopathological variables was assessed by the Pearson chi- Square ($\chi 2$) test and Fischer Exact test (FET) that was used as correction for ($\chi 2$) test when more than 20% of cells have count less than 5. To estimate *CD47* and *CD68* association with patients' survival, Kaplan–Meier curves were constructed, and the log rank test was performed for the statistical comparison of two groups. For multivariate analysis, Cox regression analysis was used to calculate predictors affecting OS and DFS with calculation of hazard ratio.

Ethical considerations

This study was conducted upon approval of the committed Institutional Research Board (IRB) at Faculty of Medicine, Mansoura University, Egypt (Code Number: MD.20.10.372.2020). Pathology code numbers of paraffin blocks were used instead of patients' names to ensure confidentiality and anonymity. All procedures followed the current revision of Helsinki Declaration of medical research involving human subjects [15]. Finally, the donor blocks were returned to archive for any additional patient's or investigative use.

Results

High expression of *CD47* was detected in 89 cases (44.5%) (Figure 1 B), while 111 cases (55.5%) showed low *CD47* expression (Figure 2 B). Concerning *CD68*, the median of *CD68* positive macrophages per high power field was 21 (range from 5 to 37). High *CD68*+TAMs infiltration was present in 79 cases (39.5%) (Figure 1 C), and 121 cases (60.5%) showed low *CD68*+ TAMs infiltration (Figure 2 C). As demonstrated in (Table 1), *CD47* expression showed statistically significant association with distant metastasis (P=0.04), as there was detected high expression of *CD47* in 58.8% of cases that developed distant metastasis compared to 41.6% of cases that are not associated with distant metastasis. There were also statistically significant associations between *CD47* high expression and advanced TNM stage (P=0.02), ER & PR negativity (P=0.04 & 0.004 respectively), and molecular subtype (P=0.03) with higher levels of *CD47* high expression in luminal B; *HER2*+ve, *HER2*-enriched & triple negative cases, compared to luminal A or luminal B; *HER2*-ve cases. There were no observed associations between *CD47* expression and other clinicopathological parameters.

CD68 was significantly associated with tumor grade (P≤ 0.001), as there was detected higher infiltration by *CD68*+TAMs in grade 3 tumor compared to grade 1 and grade 2 tumors (70.2%, 25% and 30.2%). *CD68* expression was also higher in ER, PR-negative tumors, and tumors with high KI67 proliferation index with statistically significant relations (P≤ 0.001, ≤ 0.001 & =0.01). As regards molecular subtype, there was a tendency for *CD68* high expression in Triple-negative and Her2-enriched cases compared to luminal A, luminal B; *HER2*-ve and luminal B; *HER2*+ve cases with statistically significant relation (P≤ 0.001). Meanwhile, no significant associations were found between *CD68* expression and other clinicopathological parameters.

There was a significant association between *CD47* and *CD68* expression in studied cases (P=0.002). 58.2% of cases with high *CD68*+TAMs exhibited high *CD47* expression, while 64.5% of cases with low *CD68*+ TAMs showed low *CD47* expression. Combined high expression of *CD47* and *CD68* was found in 23% of cases (Table 2). shows the association between combined expression of *CD47* and *CD68* and different clinicopathological parameters. There were significant associations between combined high expression of *CD47*, *CD68* and high tumor grade (P=0.003), advanced T stage (P=0.04), presence of distant metastasis (P=0.002), advanced TNM stage (P=0.02), ER, PR status (P ≤ 0.001), triple negative molecular subtype (P ≤ 0.001).

During the follow up period, 52 patients (26%) had relapse, and 67 patients (33.5%) died due to disease related factors. Univariate and multivariate analyses of clinicopathologic factors affecting the 5-years DFS and OS rates are shown in (Table 3). As regards DFS, univariate analysis showed significant association between shorter DFS and large tumor size (P=0.02), presence of skin invasion, stage *T4* (P≤ 0.001), presence of distant metastasis (P≤ 0.001), advanced TNM stage (P≤ 0.001), local recurrence (P≤ 0.001), triple negative tumors (P=0.01), high *CD68*+TAMs (P=0.03) (Figure 3c), and combined high expression of *CD47* and *CD68* (P≤ 0.001) (Figure 3e). There was a tendency of tumors with high expression of *CD47* to be associated with lower DFS compared to those with low *CD47* expression. However, this tendency has not reached the level of statistical significance (P=0.06) (Figure 3a). A multivariate Cox regression analysis revealed that distant metastasis and local recurrence were found to be independent prognostic predictors for lower DFS (P≤ 0.001).

Concerning OS, univariate survival analysis showed significant association between shorter OS and larger tumors (P=0.046), higher tumor grade (P=0.045), presence of skin invasion, stage *T4* (P≤ 0.001), presence of distant metastasis (P≤ 0.001), advanced TNM stage (P≤ 0.001), local recurrence (P=0.04), *ER & PR*-negative tumors (P≤ 0.001), tumors with high *Ki67* proliferation index (P=0.03), triple negative tumors (P≤ 0.001), high *CD47* expression (P=0.004) (Figure 3b), high *CD68*+TAMs (P=0.002) (Figure 3d), and combined high expression of *CD47* and *CD68* (P≤ 0.001) (Figure 3f). Multivariate analysis reported that tumor size >5 cm (P=0.04), presence of distant metastasis (P=0.01), TNM stage IV (P=0.02), local recurrence (P=0.01), and combined high expression of *CD47* and *CD68* (P=0.002) are considered independent prognostic predictors for lower OS in breast cancer patients.

Discussion

CD47 has been considered as a biomarker of several carcinomas, and its high expression is a poor clinical prognostic factor. However, the exact role of *CD47* in the prognosis of breast cancer remains unclear [7]. In this cohort study, we evaluated *CD47* IHC expression in 200 Egyptian breast cancer cases, in addition to measuring the density of TAMs infiltration by *CD68* IHC staining. The frequency of *CD47* high expression in the studied cases was 44.5%, that is close to Kosaka et al. [16] who reported overexpression of *CD47* in 36.7% of their studied cases [16]. However, studies by Yuan et al. [7] and Sun et al. [8] reported higher rates of *CD47* high expression (64.5% & 61.3% respectively) [7,8]. This discrepancy could be attributed to the differences in sample size, the scoring methods, as well as different antibody clones.

There was a statistically significant positive association between *CD47* high expression and the presence of distant metastasis, and advanced TNM stage. That agreed with

Table 1. Associations between the Expression of *CD47, CD68*, and Different Clinicopathologic Parameters.

Clinicopathological parameters	Total 200	*CD47* expression		Test of significance	*CD68*+TAMs infiltration		Test of significance
		Low (n=111)	High (n=89)		Low (n=121)	High (n=79)	
Age (years)							
≤ 50 years	72	40 (55.6%)	32 (44.4%)		46 (63.9%)	26 (36.1%)	
> 50 years	128	71 (55.5%)	57 (44.5%)	P=0.99	75 (58.6%)	53 (41.4%)	P=0.46
Size							
≤ 2 cm	30	13 (43.3%)	17 (56.7%)		17 (56.7%)	13 (43.3%)	
> 2: ≤ 5 cm	139	82 (59%)	57 (41%)	P=0.26	84 (60.4%)	55 (39.6%)	P=0.82
> 5 cm	31	16 (51.6%)	15 (48.4%)		20 (64.5%)	11 (35.5%)	
Histopathological type							
Invasive duct carcinoma, NST	156	80 (51.3%)	76 (48.7%)		88 (56.4%)	68 (43.6%)	
Invasive lobular carcinoma	22	15 (68.2%)	7 (31.8%)	P=0.12	18 (81.8%)	4 (18.2%)	P=0.07
Mucinous carcinoma	10	9 (90%)	1 (10%)		9 (90%)	1 (10%)	
Metaplastic carcinoma	5	3 (60%)	2 (40%)		2 (40%)	3 (60%)	
Micropapillary carcinoma	7	4 (57.1%)	3 (42.9%)		4 (57.1%)	3 (42.9%)	
Tumor grade							
1	4	3 (75%)	1 (25%)		3 (75%)	1 (25%)	
2	149	86 (57.7%)	63 (42.3%)	P=0.51	104 (69.8%)	45 (30.2%)	P≤ 0.001*
3	47	22 (46.8%)	25 (53.2%)		14 (29.8%)	33 (70.2%)	
Skin invasion							
Absent	193	108 (56%)	85 (44%)		119 (61.7%)	74 (38.3%)	
Present	7	3 (42.9%)	4 (57.1%)	P=0.71	2 (28.6%)	5 (71.4%)	P=0.08
Lympho-vascular emboli							
Absent	46	24 (52.2%)	22 (47.8%)		31 (67.4%)	15 (32.6%)	
Present	154	87 (56.5%)	67 (43.5%)	P=0.61	90 (58.4%)	64 (41.6%)	P=0.28
T stage							
T1	30	13 (43.3%)	17 (56.7%)		17 (56.7%)	13 (43.3%)	
T2	137	81 (59.1%)	56 (40.9%)	P=0.39	84 (61.3%)	53 (38.7%)	P=0.26
T3	26	14 (53.8%)	12 (46.2%)		18 (69.2%)	8 (30.8%)	
T4	7	3 (42.9%)	4 (57.1%)		2 (28.6%)	5 (71.4%)	
N stage							
N0	62	29 (46.8%)	33 (53.2%)		35 (56.5%)	27 (43.5%)	
N1	59	36 (61%)	23 (39%)	P=0.17	38 (64.4%)	21 (35.6%)	P=0.50
N2	50	26 (52%)	24 (48%)		33 (66%)	17 (34%)	
N3	29	20 (69%)	9 (31%)		15 (51.7%)	14 (48.3%)	
M stage (Metastasis)							
M0 (absent)	166	100 (59.5%)	68 (40.5%)	P=0.04*	105 (62.5%)	63 (37.5%)	P=0.32
M1 (present)	32	11 (34.4%)	21 (65.6%)		16 (50%)	16 (50%)	
TNM stage							
I	6	5 (83.3%)	1 (16.7%)		4 (66.7%)	2 (33.3%)	
II	93	63 (67.7%)	30 (32.3%)	P=0.02*	59 (63.4%)	34 (36.6%)	P=0.62
III	69	32 (46.4%)	37 (53.6%)		42 (60.9%)	27 (39.1%)	
IV	32	11 (34.4%)	21 (65.6%)		16 (50%)	16 (50%)	
Local recurrence							
Absent	180	100 (55.6%)	80 (44.4%)		111 (61.7%)	69 (38.3%)	
Present	20	11 (55%)	9 (45%)	P=0.96	10 (50%)	10 (50%)	P=0.31
ER							
Negative	34	14 (41.2%)	20 (58.8%)		7 (20.6%)	27 (79.4%)	
Positive	166	97 (58.4%)	69 (41.6%)	P=0.04*	114 (68.7%)	52 (31.3%)	P≤ 0.001*
PR							
Negative	48	18 (37.5%)	30 (62.5%)		16 (33.3%)	32 (66.7%)	
Positive	152	93 (61.2%)	59 (38.8%)	P=0.004*	105 (69.1%)	47 (30.9%)	P≤ 0.001*

P, Probability value; *, statistically significant (P<0.05); NST, carcinoma of no special type; CD47, cluster of differentiation 47; CD68, cluster of differentiation 68; TAMs, Tumor-associated macrophages.

Table 1. Continued

Clinicopathological parameters	Total 200	CD47 expression		Test of significance	CD68+TAMs infiltration		Test of significance
		Low (n=111)	High (n=89)		Low (n=121)	High (n=79)	
HER2							
Negative	142	85 (59.9%)	57 (40.1%)		84 (59.2%)	58 (40.8%)	
Positive	58	26 (44.8%)	32 (55.2%)	P=0.06	37 (63.8%)	21 (36.2%)	P=0.06
Ki-67 proliferation index							
Low	75	43 (57.3%)	32 (42.7%)		54 (72%)	21 (28%)	
High	125	68 (54.4%)	57 (45.6%)	P=0.69	67 (53.6%)	58 (46.4%)	P=0.01*
Molecular subtype							
Luminal A	57	32 (56.1%)	25 (43.9%)		41 (71.9%)	16 (28.1%)	
Luminal B, HER2 -ve	70	48 (68.6%)	22 (31.4%)	P=0.03*	50 (71.4%)	20 (28.6%)	P≤ 0.001*
Luminal B, HER2 +ve	39	17 (43.6%)	22 (56.4%)		23 (59%)	16 (41%)	
HER2-enriched	19	9 (47.4%)	10 (52.6%)		4 (21.1%)	15 (78.9%)	
Triple negative	15	5 (33.3%)	10 (66.7%)		3 (20%)	12 (80%)	
CD68+TAMs infiltration							
Low	121	78 (64.5%)	43 (35.5%)				
High	79	33 (41.8%)	46 (58.2%)	P=0.002*			

P, Probability value; *, statistically significant (P<0.05); NST, carcinoma of no special type; CD47, cluster of differentiation 47; CD68, cluster of differentiation 68; TAMs, Tumor-associated macrophages.

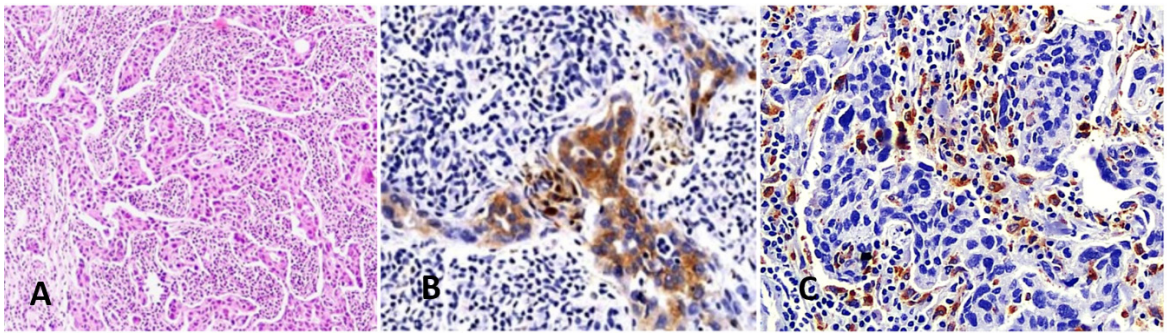

Figure 1. A Case of High-Grade Carcinoma with Medullary Pattern. H&E x100 (A), High *CD47* Expression in Tumor Cells (B) (IHC, DAB x200), High infiltration by CD68+TAMs (C) (IHC, DAB x200).

Yuan et al. [7] and Sun et al. [8] studies. Furthermore, there were also significant associations between *CD47* high expression and ER, PR negativity. As regards molecular subtypes, high expression of *CD47* was more often found in triple negative, luminal B; *HER2*+ve, and *HER2*-enriched tumors. Luminal A and luminal B; *HER2*-ve tumors showed lower expression of *CD47*. These findings These findings matched to great extent with what was reported by Chen et al. [2] and Yuan et al. [7]. On the contrary, no significant associations were found between *CD47* expression and other clinicopathological parameters.

In the current study, high infiltration by *CD68*+TAMs was detected in 39.5% of cases, and this was close to the study of Khalili et al. [17] who reported high expression of *CD68* in 38% of their cases [17]. On the contrary, Yuan et al. [7] reported a much higher level of *CD68* high expression (in 72.4% of cases) [7], and Chen et al. [2]

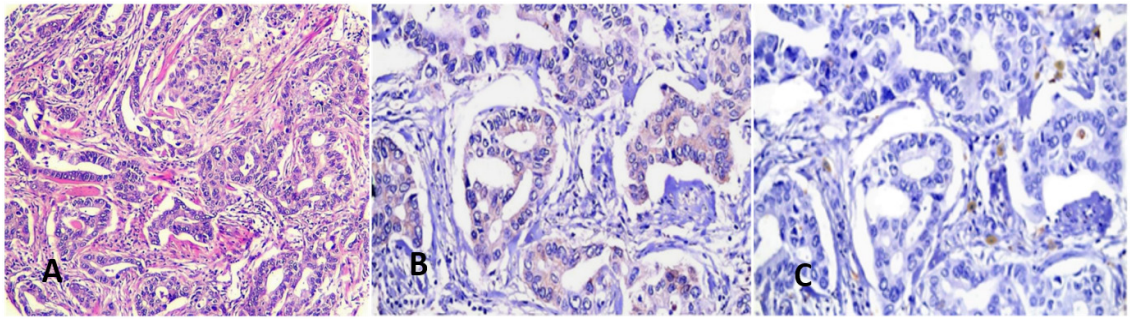

Figure 2. A Case of Grade 2 Invasive Duct Carcinoma, NST. H&E x100(A). Low *CD47* expression in tumor cells (B) (IHC, DAB x200). Low infiltration by CD68+TAMs (C) (IHC, DAB x200).

Table 2. Associations between Combined Expression of *CD47* and *CD68*, and Different Clinicopathological Parameters.

Clinicopathological parameters	Total -200	CD 47 expression		Test of significance
		Low (n=111)	High (n=89)	
Age (years)				
≤ 50 years	72	40 (55.6%)	32 (44.4%)	
> 50 years	128	71 (55.5%)	57 (44.5%)	P=0.99
Size				
≤ 2 cm	30	13 (43.3%)	17 (56.7%)	
> 2: ≤ 5 cm	139	82 (59%)	57 (41%)	P=0.26
> 5 cm	31	16 (51.6%)	15 (48.4%)	
Histopathological type				
Invasive duct carcinoma, NST	156	80 (51.3%)	76 (48.7%)	
Invasive lobular carcinoma	22	15 (68.2%)	7 (31.8%)	P=0.12
Mucinous carcinoma	10	9 (90%)	1 (10%)	
Metaplastic carcinoma	5	3 (60%)	2 (40%)	
Micropapillary carcinoma	7	4 (57.1%)	3 (42.9%)	
Tumor grade				
1	4	3 (75%)	1 (25%)	
2	149	86 (57.7%)	63 (42.3%)	P=0.51
3	47	22 (46.8%)	25 (53.2%)	
Skin invasion				
Absent	193	108 (56%)	85 (44%)	
Present	7	3 (42.9%)	4 (57.1%)	P=0.71
Lympho-vascular emboli				
Absent	46	24 (52.2%)	22 (47.8%)	
Present	154	87 (56.5%)	67 (43.5%)	P=0.61
T stage				
T1	30	13 (43.3%)	17 (56.7%)	
T2	137	81 (59.1%)	56 (40.9%)	P=0.39
T3	26	14 (53.8%)	12 (46.2%)	
T4	7	3 (42.9%)	4 (57.1%)	
N stage				
N0	62	29 (46.8%)	33 (53.2%)	
N1	59	36 (61%)	23 (39%)	P=0.17
N2	50	26 (52%)	24 (48%)	
N3	29	20 (69%)	9 (31%)	
M stage (Metastasis)				
M0 (absent)	166	100 (59.5%)	68 (40.5%)	P=0.04*
M1 (present)	32	11 (34.4%)	21 (65.6%)	
TNM stage				
I	6	5 (83.3%)	1 (16.7%)	
II	93	63 (67.7%)	30 (32.3%)	P=0.02*
III	69	32 (46.4%)	37 (53.6%)	
IV	32	11 (34.4%)	21 (65.6%)	
Local recurrence				
Absent	180	100 (55.6%)	80 (44.4%)	
Present	20	11 (55%)	9 (45%)	P=0.96
ER				
Negative	34	14 (41.2%)	20 (58.8%)	
Positive	166	97 (58.4%)	69 (41.6%)	P=0.04*

P, Probability value; *, statistically significant (P<0.05); NST, carcinoma of no special type; CD47, cluster of differentiation 47; CD68, cluster of differentiation 68.

Table 2. Continued

Clinicopathological parameters	Total -200	*CD 47* expression		Test of significance
		Low (n=111)	High (n=89)	
PR				
Negative	48	18 (37.5%)	30 (62.5%)	
Positive	152	93 (61.2%)	59 (38.8%)	P=0.004*
HER2				
Negative	142	85 (59.9%)	57 (40.1%)	
Positive	58	26 (44.8%)	32 (55.2%)	P=0.06
Ki-67 proliferation index				
Low	75	43 (57.3%)	32 (42.7%)	
High	125	68 (54.4%)	57 (45.6%)	P=0.69
Molecular subtype				
Luminal A	57	32 (56.1%)	25 (43.9%)	
Luminal B, *HER2* -ve	70	48 (68.6%)	22 (31.4%)	P=0.03*
Luminal B, *HER2* +ve	39	17 (43.6%)	22 (56.4%)	
HER2-enriched	19	9 (47.4%)	10 (52.6%)	
Triple negative	15	5 (33.3%)	10 (66.7%)	

P, Probability value; *, statistically significant (P<0.05); NST, carcinoma of no special type; CD47, cluster of differentiation 47; CD68, cluster of differentiation 68.

stated that only 24% of their cases showed high counts of *CD68*+TAMs [2]. This wide variation of *CD68* expression may be attributed to the differences in the interpretations of the staining pattern, the scoring methods, the adopted cut-off value, as well as the different monoclonal antibodies used by the investigators.

The present study showed a statistically significant association between *CD68* expression and high tumor grade. This finding comes in agreement with several other studies that also reported this significant association [2,7,18-23]. On the contrary, few other studies didn't report such association [5,17]. It was proposed that high-grade tumors may elaborate higher levels of cytokines that recruit and modulate macrophages as monocyte colony-stimulating factors, interleukin-10, and/or transforming growth factor-β resulting in increased density of TAMs within high-grade tumors [24]. Moreover, TAMs may secrete different cytokines and growth factors that provide mitogenic signals to malignant cells [25].

This study disclosed that the density of *CD68*+TAMs had negative associations with ER, PR status and positive association with KI67 proliferation index, and these were statistically significant. In support to our observations, other studies reported that *CD68*+TAMs were significantly associated with hormonal receptor negativity and high *Ki67* proliferation [18,20,21,26]. However, no significant association was found between *CD68* expression and *HER2* status. It is worth noting that the association between *HER2* and TAMs remains undetermined among studies. The present study revealed a significant association between *CD68* expression and hormone receptor-negative tumors (*HER2*-enriched and triple negative molecular subtypes), and this finding is in accordance with several other studies [2,7,20,22,27].

We reported significant positive association between *CD47* and *CD68* expression in studied cases. This is in line with Chen et al. [2] and Yuan et al. [7]. In this study, there were detected significant associations between *CD47*high/*CD68* high cases and high tumor grade, advanced T stage, presence of distant metastasis, advanced TNM stage. and ER and PR status. Furthermore, *CD47*high/*CD68*high occurred more frequently in triple negative tumors compared to other molecular subtypes. These findings match to great extent with the previous studies that also assess the associations between clinicopathological variables and combined expression of *CD47* and *CD68* [2,7].

In our study, *CD47* high expression was found to predict poor OS despite lacking significant association with DFS, but it is not considered as independent poor prognostic factor by multivariate analysis. Similarly, Sun et al. [8] reported that patients with *CD47* high expression had significantly shorter OS compared with patients with *CD47* low expression [8]. Yuan et al. [7] reported that high *CD47* expression in breast cancer tissues had a limited association with reduced DFS, and that it was not an independent predictor of poor DFS [7]. Regarding the impact of *CD68*+TAMs on patient's survival, the current study reported significant positive association between *CD68*+ TAMs and shorter DFS and OS periods. However, by multivariate analysis, *CD68* expression is not considered as independent poor prognostic factor for breast carcinoma patients. Similar findings were reported by Medrek et al. [27] who found that the density of *CD68*+ macrophages in tumor stroma correlated with poor OS and poor DFS, but it was not an independent prognostic factor [27]. In the same vein, Yuan et al. [7] showed a correlation between high expression of *CD68* and reduced DFS, but a limited prognostic value of *CD68* expression was observed based on the multivariate Cox proportional analysis [7].

The present study unveiled a significant association between *CD47*high/*CD68*high tumors and shorter DFS

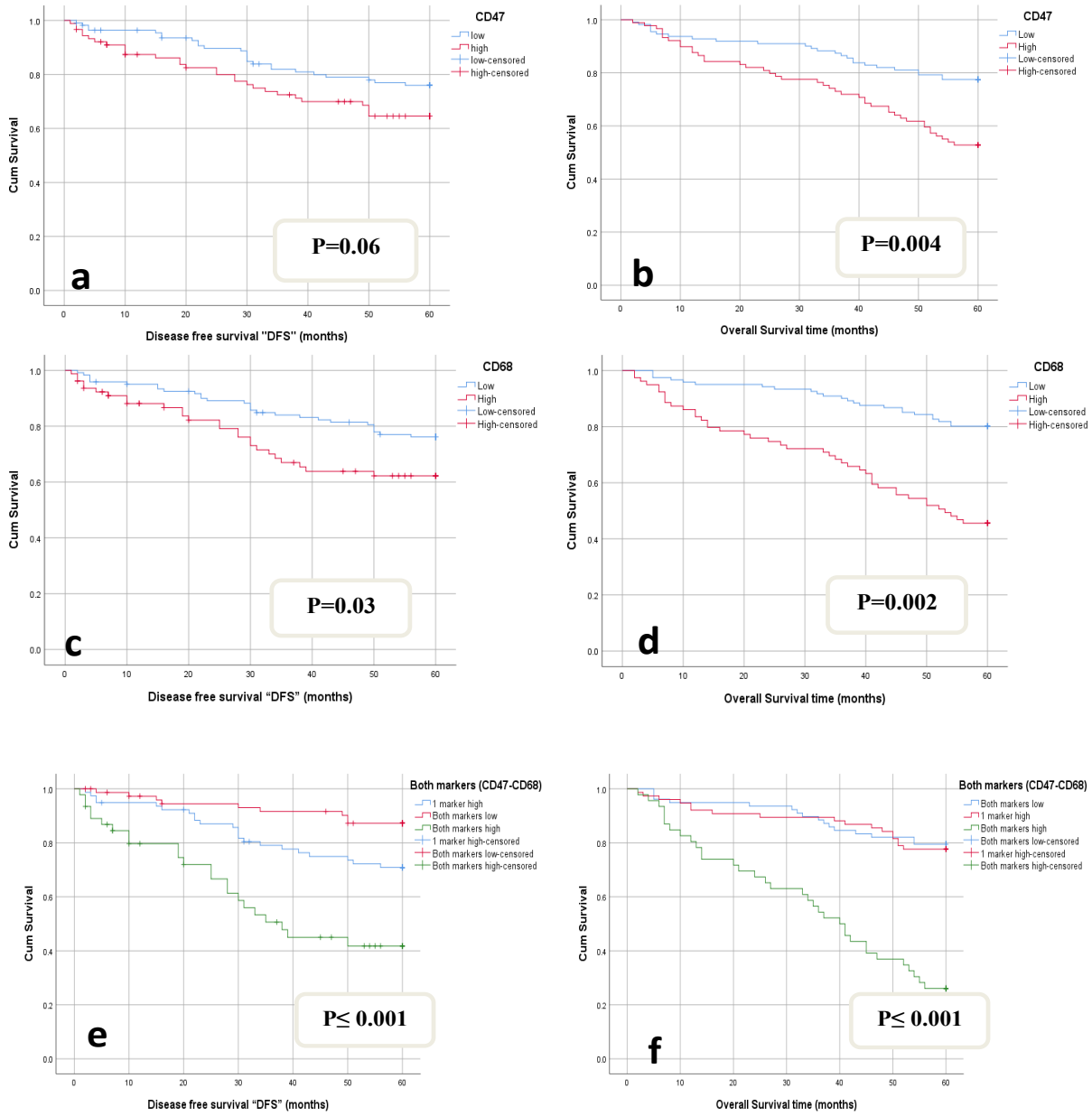

Figure 3. Kaplan-Meir Survival Curves for Patients with Breast Carcinoma Stratified by *CD47* Expression, *CD68* Expression, and Combined *CD47/CD68* Expression. No statistically significant association between *CD47* and DFS (a; log rank; p= 0.06). Significantly lower overall survival (OS) in patients with high *CD47* expression compared to those with low *CD47* expression (b; log rank; p = 0.004). Significantly lower DFS (c; log-rank; p= 0.03) and OS (d; log-rank; p=0.002) in patients with high infiltration by *CD68*+TAMs compared to patients with low infiltration by *CD68*+TAMs. Significantly lower DFS (e; log-rank; p≤ 0.001) and OS (f; log-rank; p≤ 0.001) in patients with combined high expression of *CD47* and *CD68* than in other patients

and OS periods. Moreover, combined high expression of *CD47* and *CD68* was found to be an independent prognostic factor for shorter OS periods and increased risk of mortality in breast carcinoma patients. These data suggested that the prognostic value of combined high expression of *CD47* and *CD68* is better than that of *CD47* or *CD68* alone. In conclusion, our study indicated that *CD47* high expression is related to poor prognosis in breast cancer patients, and when combined with high infiltration by *CD68*+TAMs it represents an independent prognostic factor for poor overall survival. Therefore, *CD47* is a promising prognostic and therapeutic target in breast carcinoma that may direct selection of patients for immunotherapy.

Author Contribution Statement

All authors contributed equally, all authors reviewed the results and approved the final version of the manuscript.

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None.

Table 3. Univariate and Multivariate Survival Analysis of the Disease-Free Survival (DFS) and Overall Survival (OS) in breast Carcinoma.

Clinicopathological parameters	Disease-free survival				Overall survival			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	Median DFS time (min-max) / months	Log-rank test	Hazard ratio (95% CI)	P value	Median OS time (min-max) per months	Log-rank test	Hazard ratio (95% CI)	P value
Age								
≤ 50 years	51 (47.1 – 54.8)				52.1 (48.6 – 55.6)			
> 50 years	49.2 (45.8 – 52.6)	P=0.93			48.4 (45.2 – 53.7)	P=0.39		
Size								
≤ 2 cm (r)	52.6 (42.6 – 58.7)				51.2 (43.4 – 58.9)			
> 2: ≤ 5 cm	51.4 (41.6 – 54.1)	P=0.02*	0.85 (0.03 – 22.7)	0.92	49.4 (42.8 – 56.1)	P=0.046*	1.7 (0.15 – 21.1)	0.66
> 5 cm	40.3 (31.8 – 48.8)		2.3 (0.61 – 8.4)	0.22	40.8 (36.6– 50.9)		3.2 (1.1 – 9.5)	0.04*
Histopathological type								
Invasive duct carcinoma, NST	ND				49.8 (47.1 – 52.6)			
Invasive lobular carcinoma		P=0.69			49.8 (42.7 – 56.9)	P=0.89		
Mucinous carcinoma					53.8 (44.2 – 63.4)			
Metaplastic carcinoma					40.4 (19.2 – 60)			
Micropapillary carcinoma					49.1 (34.4 – 64)			
Tumor grade								
1	51.4 (46.3 – 56.6)				51.7 (49.2 – 58.2)			
2	49.4 (37.5 – 52.4)	P=0.83			46.3 (39.9 – 69.6)	P=0.045*	0.38 (0.05 – 3.1)	0.37
3	46 (22.3 – 49.8)				40.8 (37.7 – 49.9)		0.47 (0.05 – 4.1)	0.49
Skin invasion								
Absent (r)	50.8 (48.3– 53.3)				50.7 (48.3 – 53.1)			
Present	15.7 (5.8 – 25.7)	P≤ 0.001*	1.6 (0.39 – 6.4)	0.52	23.1 (8.7 – 37.6)	P≤ 0.001*	2.3 (0.72 – 7.3)	0.16
Lymphovascular invasion								
Absent	51.3 (46.3 –56.4)				48.7 (43.3 – 54)			
Present	49.4 (46.4 – 52.3)	P=0.46			50.1 (47.3 – 52.8)	P=0.58		
T stage								
T1 (r)	52.6 (46.6 – 58.7)				51.5 (48.7 – 59.2)			
T2	51.6 (48.9 – 54.3)	P≤ 0.001*	1.5 (0.05 – 39.9)	0.83	49.4 (42.8 – 56.1)	P≤ 0.001*	1.1 (0.11 – 12.2)	0.91
T3	44.5 (36 – 53.1)		2.6 (0.67 – 23.7)	0.65	48.3 (41.7 – 54.9)		0.56 (0.43 – 9.3)	0.87
T4	15.7 (5.8 – 25.7)		3.1 (0.08 – 11.9)	0.53	23.1 (8.7 – 37.6)		2.4 (0.51 – 13.5)	0.32

Table 3. Continued

Clinicopathological parameters	Disease-free survival				Overall survival			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	Median DFS time (min-max) / months	Log-rank test	Hazard ratio (95% CI)	P value	Median OS time (min-max) per months	Log-rank test	Hazard ratio (95% CI)	P value
N stage								
N0	52.7 (48.4 – 56.9)				53.1 (49.5 – 56.6)			
N1	49.5 (45 – 53.9)	P=0.52			50.7 (46 – 55.4)	P=0.17		
N2	48.6 (43.1 – 54.1)				51.7 (47.4 – 56.1)			
N3	42.9 (39.9 – 53.9)				47.9 (41.8 – 54.2)			
M stage (Metastasis)								
M0 (r)	55.8 (54 – 57.7)				53 (50.6 – 55.4)			
M1	22 (16.1 – 28)	P≤ 0.001*	38.6 (24.6 – 80.5)	≤ 0.001*	33.9 (27.9 – 39.8)	P≤ 0.001*	9.1 (1.6 – 51.8)	0.01*
TNM stage								
I (r)	ND				54.8 (48.9 – 61.7)			
II		P≤ 0.001*	18.8 (0.01 – 100.1)	0.95	52.9 (49.6 – 56.1)	P≤ 0.001*	0.36 (0.09 – 1.5)	0.09
III			20.2 (0.04 – 33.5)	0.94	47.5 (50.1 – 56.9)		0.44 (0.09 – 2.1)	0.08
IV			3.3 (0.02 – 19.1)	0.98	33 (26.8 – 39.1)		1.8 (1.2 – 6.7)	0.02*
Local recurrence								
Absent	52.5 (49.9 – 55)				50.2 (47.5 – 52.8)			
Present	28.4 (23.6 – 33.1)	P≤ 0.001*	24.9 (13.2 – 61.9)	≤ 0.001*	46.2 (39.3 – 53.1)	P=0.04*	2.9 (1.3 – 6.6)	0.01*
ER								
Negative (r)	42.1 (39.8 – 51.4)				37.9 (30.2 – 45.7)			
Positive	50.2 (47.5 – 52.9)	P=0.44			52.2 (49.9 – 54.5)	P≤ 0.001*	0.62 (0.11 – 3.7)	0.59
PR								
Negative (r)	41.9 (38.5 – 50.3)				38.5 (35.3 – 51.9)			
Positive	49.9 (47 – 52.9)	P=0.53			51.9 (46.9 – 54.8)	P≤ 0.001*	1.1 (0.4 – 3.1)	0.83
HER2								
Negative	50.7 (47.7 – 53.6)				50.4 (47.5 – 53.3)			
Positive	43.8 (39.9 – 50.7)	P=0.24			48.2 (43.6 – 52.9)	P=0.27		
KI-67 proliferation index (r)								
Low	52.1 (48.4 – 55.9)				52.6 (49.1 – 56.2)			
High	45.4 (40 – 50.8)	P=0.13			42 (40.8 – 51.3)	P=0.03*	1.7 (0.49 – 6.3)	0.39

Table 3. Continued

Clinicopathological parameters	Disease-free survival				Overall survival			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	Median DFS time (min-max) / months	Log-rank test	Hazard ratio (95% CI)	P value	Median OS time (min-max) per months	Log-rank test	Hazard ratio (95% CI)	P value
Molecular subtype								
Luminal A (r)	53.1 (48.8 – 57.3)				52.4 (48.1– 56.6)			
Luminal B, HER2 -ve	50.5 (46.4 – 54.7)	P=0.01*	0.82 (0.33 – 2.0)	0.66	53.4 (50.3 – 56.6)	P≤ 0.001*	0.41 (0.09 – 1.8)	0.24
Luminal B, HER2 +ve	45.7 (39.7 – 51.8)		0.65 (0.23 – 1.7)	0.37	49.6 (44.6 – 54.6)		0.67 (0.19 – 2.4)	0.53
HER2-enriched	52.9 (45.3 – 60.4)		0.86 (0.19 – 3.9)	0.84	45.4 (35.8 – 54.9)		0.64 (0.22 – 1.9)	0.41
Triple negative	37.8 (25.1 – 50.5)		0.68 (0.18 – 2.5)	0.56	28.5 (17.5 – 39.5)		0.69 (0.33 – 1.8)	0.21
CD47 expression								
Low (r)	52.2 (49.2 – 55.1)				52.9 (49.9 – 55.8)			
High	46.9 (42.6 – 51.2)	P=0.06			41.9 (39.7 – 49.9)	P=0.004*	1.2 (0.47 – 3.1)	0.71
CD68+TAMs infiltration								
Low (r)	52.4 (49.5-55.2)				54.6 (52.2 – 56.9)			
High	40.6 (36.9 – 45.3)	P=0.03*	1.6 (0.3 – 8.3)	0.58	42.4 (37.8 – 46.9)	P =0.002*	2.6 (1.1 – 6.6)	0.057
Combined CD47 & CD68 expression								
Both low (r)	56.1 (53.3 – 58.8)				54 (50.9 – 57.1)			
One high & one low	50.5 (46.7 – 54.3)	P≤ 0.001*	0.92 (0.32 – 2.6)	0.87	53.3 (49.9 – 56.8)	P≤ 0.001*	1.1 (0.55 – 2.2)	0.79
Both high	37.6 (30.9 – 44.3)		1.4 (0.08 – 3.8)	0.76	36.7 (30.8 – 42.5)		4.7 (3.1 – 9.9)	0.002*

Compliance with Ethical Standards

This study was conducted upon approval of the committed Institutional Research Board (IRB) at Faculty of Medicine, Mansoura University, Egypt (Code Number: MD.20.10.372.2020). The study was processed under the ethical standards of the Helsinki declaration.

This study wasn't approved by any scientific body and isn't part of an approved student thesis.

Availability of data

The datasets are available from the corresponding author upon request.

Conflict of interest

The authors declare that there are no conflicts of interest to disclose.

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