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Possible Association of CD68 Positive Macrophages with Some other Prognostic Factors (ki67, ER, PR, Her2 neu) in Primary Breast Cancer and Axillary Lymph Node Metastasis

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Abstract

Background: Breast cancer is formed of a neoplastic component (epithelial) and a non-neoplastic component (stroma). Stromal- stromal and tumor- stromal interactions have been shown in the regulation of cancer cell growth, metastatic capacity and outcome of treatment. Tumor-associated macrophages (TAMs) are a component of tumor stroma reactionsand are considered as an important component of breast cancer tumor tissue which approximately form 50 % to 80 % of tumor tissue.In this study, the frequency of CD68 positive cells in association with other factors such as age, tumor size, ER, PR, Ki-67, Her2-neu receptors, stage and grade in invasive carcinoma tissues were morphologically and statistically evaluated. The frequency of CD68 was also discussed in relation to the number of involved lymph nodes.

Method: A total of 50 invasive breast cancer patients with and without axillary lymph nodes involvement were studied. IHC staining for CD68 and Ki-67 markers was performed. For each tumor, 5 fields with different density of CD68 were counted under 400x by optical microscopy and the average of the five fields was taken as the percentage. Patients were divided into the two groups of low infiltration and high infiltration based on the percentage of CD68.By the same way, patients were divided into the two groups of low infiltration and high infiltration based on Ki67 percentage.

Results: CD68 positive cells had significant correlation with ER negative and higher Ki67. No significant correlation was found between CD68 positive cells and the number of involved lymph nodes, age, size, HER2neu, PR, stage and grade.

Conclusion: It seems that the presence of CD68 -positive macrophages in invasive breast cancers and nearby lymph nodes is associated with a worse prognosis.

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Introduction

Proliferation of neoplastic components of epithelial and/or stromal cells play role in breast cancer development (1-3).

Stromal-stromal and tumor- stromal interactions have been demonstrated in the regulation of cancer cell growth, metastatic capacity, locations of metastases, and outcome of treatment (4).Tumor-associated macrophages (TAMs) are a component of tumor stroma reaction and an important component of breast cancer tumor tissue composed of approximately 50 % to 80 % of tumor tissue (5).

TAMs, as the most potent anti-tumor factors that suppress the immune system, can be considered as the biggest barrier in accomplishing immunotherapy (6).

In recent years, TAMs have been suggested as the seventh sign of "Hall Mark" of cancer (7).

In several studies, adverse prognostic factor for patients with TAMs infiltration of invasive breast cancer was correlated with worse prognosis (8).

Complementary treatment regimens including anti-TAMs were tested on mice with invasive breast cancer and 62% of animals who received these regimens were completely without metastasis (9).

In this study, the frequency of CD68 cells in association with other factors such as age, tumor size, ER, PR, Ki-67, Her2-neu receptors and stage and grade in invasive carcinoma tissues was evaluated morphologically and statistically. The frequency of CD68 was also discussed in relation to the number of involved lymph nodes.

Materials and Methods

In the present study, medical files of 50 invasive breast cancer patients with and without axillary lymph nodes involvement who had pathology reports in the labs of Bahonar and Afzalipour hospitals during 2009-2015 were investigated for demographic and other pathologic findings. Important pathological and clinical data including grade, tumor size, lymph node involvements and stage, and also IHC receptors for ER, PR, and Her2Neu were collected from the patient's files.

Then, all slides were reviewed by two pathologists independently. Normal and carcinoma areas were selected along with a lymph node in the same paraffin block.

Paraffin blocks were cut in 4 micron sections for IHC staining of CD68 (DAKO code was IS609) and Ki-67 (DAKO code was IS626). They were ready to be used.

The corresponding silanized slides were prepared and then put in oven 60° C for one hour. After hydrating step and retrieval buffer solution(PH=9), they were put in microwave for 20 minutes. Then, slides were placed in washing buffer PH = 7.4 for 5 minutes in order to get cold. In the peroxidase step, slides were put in dark and wet place for 10 minutes, and then in washing buffer for 5 minutes. In the primary antibody step, specific antibody was placed on the sample for one hour, and then was placed in washing buffer for 5 minutes. After 30 minutes, secondary antibodies were

washed with washing buffer for 5 minutes. In DAB step, after 5-15 minutes, they were washed by water. In the next step, nucleolus was stained by hematoxylin and then washed by water. The final stages were dehydrating, clearing and mounting by cover slides.

For each tumor and lymph node, separately, 5 fields with different density of CD68 were counted by optical microscopy under 400x and the average of the five fields was reported as percentage. Stromal infiltrations of TAMs were diffused sparsely.

Patients were divided into the two groups according to the percentage of CD68 positive cells; group 1 consisted of 31 patients with low infiltration (5-39%) and Group 2 consisted of 19 patients with high infiltration (40-87%)(Figures 1& 2).

Similarly, for each tumor and lymph node, separately, Ki67 was counted by optical microscopy and patients weredivided into the two groups; group 1 consisted of 35 patients with low infiltration (1-24%) and group 2consisted of 15 patients with high infiltration (25-86%) (Figure 3).

Data were analyzed through SPSS22 software and using t-test, ANOVA and Tukey test. P-value of <0.05 was considered as statistically significant level.

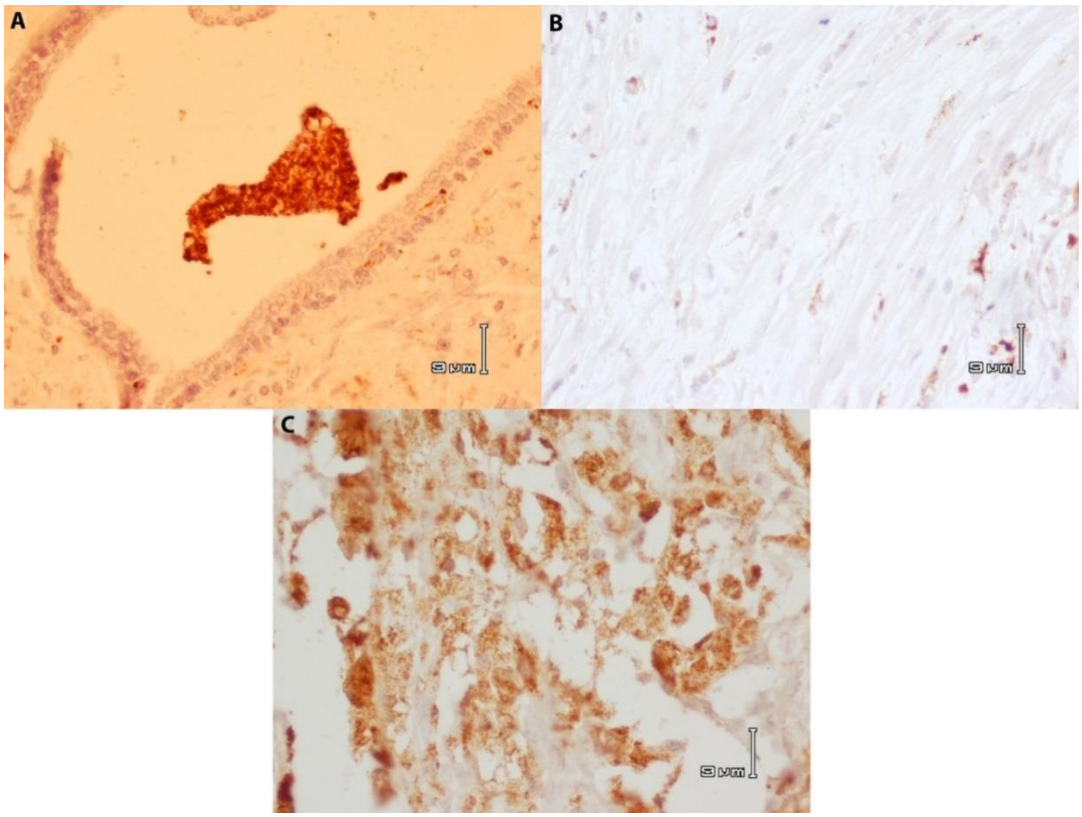

Figure 1. IHC detection of TAMs (CD68+) in normal and breast cancer tissues. CD68 expression was found in cytoplasm mainly, positive staining was brown particles. In breast cancer samples, sparsely TAMs exist in tumor stroma. A. CD68 positive macrophages in fibrocystic breast tissue (x100). B. TAMs low-infiltration in breast cancer specimen (×400). C. TAMs high-infiltration in breast cancer specimen (×400).

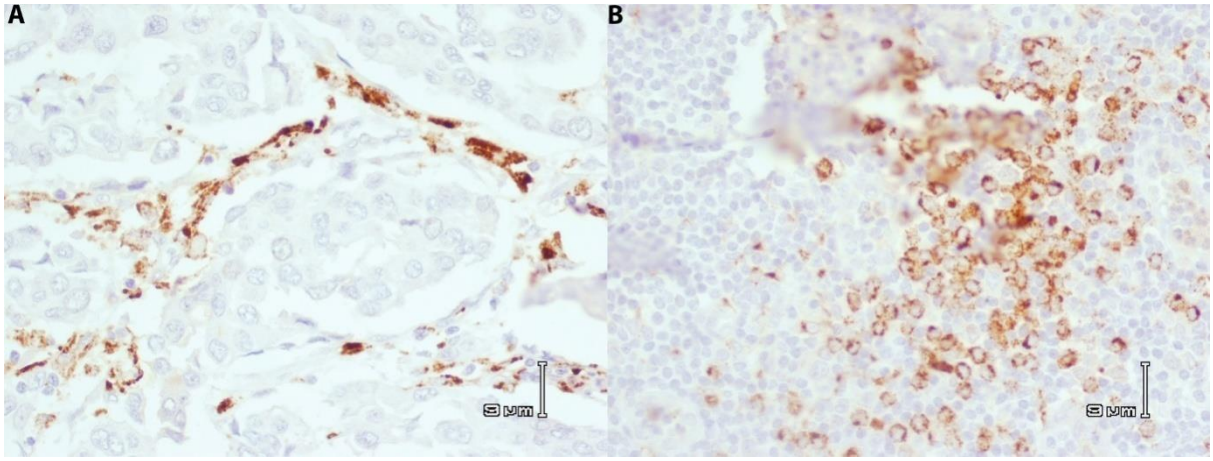

Figure 2. IHC detection of TAMs (CD68+) in lymph node tissues. In lymph node samples, diffusely scattered TAMs exist in the marginal sinus and medullary cord. A. TAMs in involved lymph node specimen (×100). B. CD68 positive cells in unaffected lymph node specimen (x100)

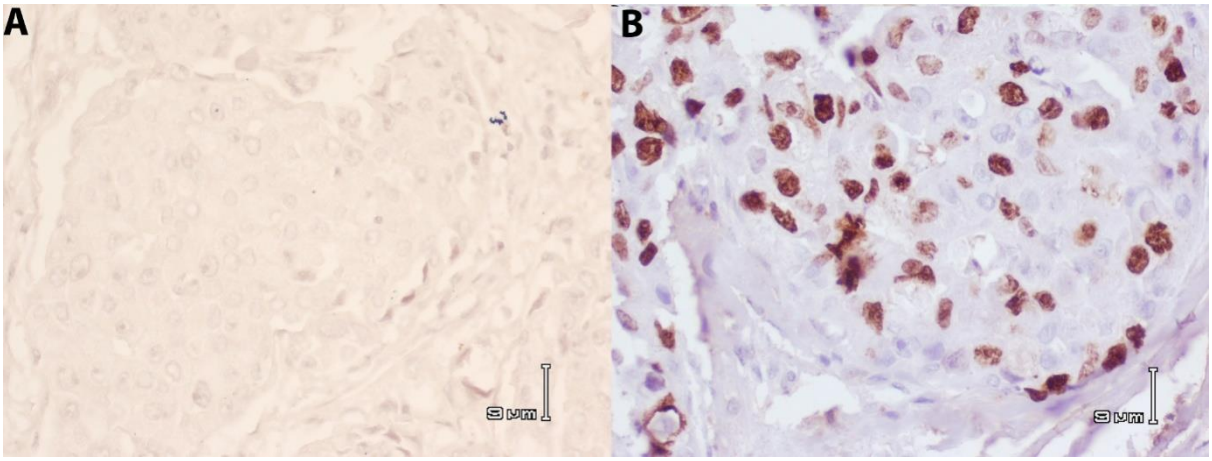

Figure 3. IHC detection of Ki67 in breast cancer tissue.Ki67 was found in nuclear cells, positive staining was deep brown particles. A. Low infiltration of Ki67 (x400). B. High infiltration of Ki67 (x400)

Results

In this study, medical files of 50 breast cancer patients with or without axillary lymph nodes involvement were investigated. Based on CD68 positive cells (TAMs), patients were divided into the group 1(low infiltration) and group 2 (high infiltration). The average density of TAMs infiltration was 32.79% in the tumor and 37.7% in the lymph nodes. In the same way, the average density of Ki67 was 1.38 % in breast tumors and 33.77% in the affected lymph nodes.

In CD68 low infiltration group (group 1), 83.9% were ER- positive and 16.1% were ER-negative and in CD68 high infiltration group (group 2), 47.4% and 52.6% were ER-positive and ER-negative respectively, which demonstrated the statistically significant relation between infiltration of CD68 positive cells and ER- negative (P value = 0.006).

In CD68 low infiltration group, 54.8% were PR positive and 45.2% were PR negative and in CD68 high infiltration group, 47.4% were PR positive and 52.6% were PR negative.

In CD68 low infiltration group, Her2Neu was negative in 38.7%, +1 in 19.4%, +2 in 25.8%,and +3 in 5 16.1% and in the CD68 high infiltration group, Her2Neuwas negative in 21.1%, +1 in 31.6%, 2+ in 26.3%,and +3 in 21.1%.

Based on Ki67 infiltration, patients were divided into the two groups of low infiltration and high infiltration. In CD68 low infiltration group, 83.9% had low infiltration of Ki67 and 16.1% had high infiltration of Ki67. Moreover, in CD68 high infiltration group, 47.4% showed low infiltration of Ki67 and 52.6% showed high infiltration of Ki67; This showed a statistically significant relationship between the infiltration of CD68 and high Ki67 (P value = 0.006).

In CD68 low infiltration group, the number of involved lymph nodes was 0-2 in 41.9%, 2-7 in 29% and 8-17 in 29%. In CD68 high infiltration group, the number of involved lymph nodes was 0 in 36.8%, 2-7 in 47.4% and 8-17 in 15.8%.

In CD68 low infiltration group, 48.4% were under 40 years and 51.6% were 41 to 80 years old and in CD68 high infiltration group, 63.2% were under 40 years and 36.8% were 41 to 80 years old.

In CD68 low infiltration group, tumor size was less than 2 cm in 9.7%, 2- 5 cm in 67.7% and greater than 5 cmin 22.6% and in CD68 high infiltration group, tumor size was less than 2 cm in 15.8%, 2-5 cm in 52.6% and greater than 5 cm in 31.6%.

In CD68 low infiltration group, the stage was IIA in 29%, IIB in 38.7%, IIIAin 16.1%, IIIBin 3.2% and IIIC in 12.9%. While, in CD68 high infiltration group, the stage was IIA in 21%, IIB in 26.3%, IIIA in 36.8%, IIIB in 0%and IIIC in15.8%.

In CD68 low infiltration group, the grade was I in6.5%, II in 61.3%and III in 32.3% and in CD68 high infiltration group, the grade was I in 28.1%, II in 47.34% and III in 31.6%.

Overall, there were negative correlation between CD68 positive cells infiltration and ER (P value = 0.006).

A positive correlation was found between CD68 positive cells infiltration and Ki67 index (P value = 0.006).

There was not any association between CD68 positive cells infiltration and PR, Her2Neu receptors, stage and grade.

There was not any relationship between CD68 positive cells infiltration and the number of involved lymph nodes The above results have been summarized in table1.

Table 1. Demographic data of high and low positive CD68 cells comparing with age , tumor size, number of involved lymph nodes, estrogen receptor (ER), progesterone receptor (PR), Her2 Neu receptor, Ki 67, grade and stage of tumor

Variables	Number of Cases	High CD68	Low CD68	P value
Age				
< & = 40	27	12	15	0.309
> 40	23	7	16	
(years)				
Tumor size				
<2	6	3	3	
>2 & <=5	31	10	21	0.558
>5	13	6	7	
(cm)				
Number of axillary lymph nodes				
0	16	6	10	
1-3	13	4	9	0.779
>3	21	9	12	
ER				
+	35	9	26	0.006
-	5	10	5	
PR				
+	26	9	17	0.608
-	24	10	14	
Her2neu				
-	16	4	12	
+1	12	6	6	0.564
+2	13	5	8	
+3	9	4	5	
Ki67				
1-24%	35	9	26	0.006
25-86%	15	10	5	
Grade				
I	6	4	2	
II	28	9	19	0.286
III	16	6	10	
Stage				
IIA	13	4	9	
IIB	17	5	12	
IIIA	12	7	5	
IIIB	1	0	1	0.461
IIIC	7	3	4	

Discussion

According to Zhang study, the frequency of tumor-associated macrophages (TAMs) is higher in the higher grades of breast cancer. In breast cancer, focal infiltrations of TAMs are directly correlated with the invasion of tumor cells, enhancement of vascularization and involvement of axillary lymph nodes. Patients with high density of TAMs have shorter survival life. Invasive breast cancer patients with higher infiltration of TAMs have worse prognosis and this marker can be used as a prognostic marker in invasive breast cancers (8).

In a study conducted by Yang et al, TAMs infiltration was much higher in the tumor tissue than in normal tissue and tumors with larger size and higher grade had higher infiltration of TAMs. They led to cancer progression with poor prognosis. However, they suggested that in relation to lymph node involvement, TAMs density in metastatic lymph nodes was lower than that in non- metastatic lymph nodes

suggesting their different role in lymph nodes which requires further studies in the future (10).

In a study conducted by Medrek et al, TAMs were mostly present in the tumor stroma, not between the tumors nests. CD68 was a marker of pan _ macrophage. CD163 was a specific marker of M2 TAMs. TAMs staining by CD163 in the stroma had positive relationship with higher grade, larger size, higher Ki67 presentation, ER negativity, PR negativity and also triple negativity (11).

In the present study, TAMs were mostly present in the tumor stroma, not inside the tumor nests. The infiltration of tumor CD68 positive cells was positively associated with Ki67 index. Furthermore, tumor infiltration of CD68 positive cells was significantly correlated with negative ER. Nevertheless, there was not any significant relationship between tumor infiltration and lymph node involvement, age, tumor's size, stage and grade, PR and Her2-Neu receptors. We found out that CD68 positive cells as a factor can worse the prognosis.

Overall, there were generally two types of macrophages: Macrophages type 1 (M1) and macrophages type 2 (M2).Unlike M1, M2 macrophages are the cause of cancer development (12). Although both types of macrophages in the tumor stroma can be found, TAMs mainly consisted of M2 (8).

However, the dual nature of the macrophages in tumor progression is called"balance of macrophages" and it has been led to the development of macrophage balance hypothesis. In other words, due to their ability in tumor progression and prevention of tumor progression, they were known as "double - edged sword" (12-19).

Metastasis to the axillary lymph nodes was the most valid factor among prognostic factors in breast cancers treatment followed by hormone receptors, HER2-neu expression, tumor size, histologic type, grade of tumor, lymph-vascular invasion, and speed of proliferation. Although, there are many prognostic factors for predicting clinical outcome of invasive breast cancers, the issue of better prognostic markers that can lead to more effective treatment has been still remained controversial (20).

In some studies, the effects of anti-TAMs were evaluated by small molecule inhibitors that had tumor suppression activity (21). For example, Yondelies anti neoplastic materials have showed cytotoxic effects on TAMs and caused significant decrease in production of IL-6 and CCL-2, which suppressed the growth of inflammatory cells associated with the tumor (22).

In another study, a combination that inhibited the secretion of MMP (matrix metalloproteinase) of TAMs could inhibit Metalloproteinase activity of tumor and reduce angiogenesis through VEGF (vascular endothelial growth factor) tyrosine kinase receptors (23).

In a different study, CCL-5 chemokine, as a key marker, was secreted by TAMs. CCL-5 chemokine antagonist reduced tumor's growth and spread significantly (24).

In order to have more precises assessment, it is better to check the specific marker of CD163 in the detection of M2 TAMs, and the frequency of this markerin relation to variables such as age, tumor size,ER, PR, Ki-67, Her2-neu receptors, stage, grade andlymph nodes involvement.

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