Frequency of regulatory B cells phenotypes in breast cancer patients in Egypt

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Abstract

Accumulating evidence has indicated that immune regulatory cells are involved in the establishment of the anti-tumor activity, however; the role of regulatory B cells (B-regs) in breast cancer (BC) remains unclear. This study intended to assess the frequency of peripheral B-regs phenotypes in patients with BC, and to determine the relation between these phenotypes and the patient's clinicopathological characters. The expressions of the immune cell populations were analyzed by four-color flow cytometry in 40 naïve BC patients and 10 age-matched apparently healthy individuals as controls attending the department of Clinical Oncology and Nuclear Medicine at Assiut University Hospitals. The percentages of B-regs phenotypes $CD19^+IL10^+$ and $CD19^+CD24^{hi}CD27^+IL10^+$ were higher in BC patients than in the controls. The percentage of $CD19^+IL10^+$ B cells phenotype was significantly associated with the HER-2 expression levels, T, and N stages of BC. In conclusion, high percentage of B-regs phenotypes $CD19^+IL10^+$ and $CD19^+CD24^{hi}CD27^+IL10^+$ in BC patients indicates a possible role in immune suppression during the development of BC.

Keywords: Breast cancer; Clinicopathological characteristics; regulatory B cell; interleukin-10.

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Introduction

In Egypt, the National Cancer Institute reported that breast cancer (BC) is the most common site of cancer in women and accounts for about 38.8% of total malignancies among Egyptian females.[1] BC is the most common malignancy in women in the United States and is second only to lung cancer as a cause of cancer death. [2] The risk of developing breast cancer throughout life is 12%.[3]

The role of the immune system in prevention of tumors has been studied over years. It could specially identify and eliminate tumor cells through the tumor-associated antigens (TAA) or molecules expressed by the tumor cells. This process was described as tumor immune surveillance or tumor immunoediting. [4]

The role of T-cell-mediated immune responses in solid tumors is well established and has become the driving force for major therapeutic advances. In contrast, the knowledge of B-cell biology in cancer is less well developed.[5.]

B cells can potentially inhibit the development and progression of cancers by making antitumor antibodies or by differentiating into appropriate effector B-cell states. B-cell-derived antibodies play an essential role in protection against tumor development by helping to clear oncogenic viruses before they can become established and initiate tumor development. An excellent example of this is the use of human papillomavirus (HPV) vaccines to prevent cervical cancer.[6, 7]

However, in 2002, Mizoguchi and collaborators discovered a subset of B cells called B-regulatory cells (B-regs), identified as IL-10 producing B cells.[8] B-regs perform immunosuppressive functions by responding to signals from the tumor microenvironment that promote tumor growth via interactions with different immune cells as T regulatory cells (T-regs), tumor-associated macrophages, CD4^{+} and CD8^{+} T lymphocytes, myeloid-derived suppressor cells (MDSC), natural killer (NK) cells, and enhancing down-regulatory immune functions by production of IL-10 and/or transforming growth factor beta (TGF-β).[7, 9]

There are no specific markers to identify different phenotypes of B-regs, but most of human B-regs were found to be originated from transitional CD24^{hi}CD38^{hi} and memory CD24^{hi}CD27^{+} B cells.[10] Although, there are different markers used in identification of B-regs, but IL-10 secretion is still a vital marker.[11] B regulatory cells could suppress the proliferation of CD4^{+}T cells and enhance FoxP3 and cytotoxic T-lymphocyte antigen 4 (CTLA-4) expressions in T-reg cells by producing IL-10 and TGF- β. B-regs did not belong to any clearly defined B cell subsets but they added value in both the CD27^{+} and the CD38^{hi} compartments.[10,12]

Although most B-regs studies have focused on autoimmune mouse models and patients with autoimmune diseases, several mouse studies of cancer have also revealed the existence of protumorigenic B-regs.[13]

At present, the subset composition and function of B-regs in human cancer including BC are largely unclear. Also, there was no previous study in Egypt detecting B-regs phenotypes in BC patients. Therefore, we undertook the present study to evaluate the frequency of peripheral B-regs in BC patients in Egypt. Also,

to determine the relation between B-regs phenotype and the clinicopathological characters of BC patients.

Subjects and Methods

Study design

This case-control study was carried out at the Medical Research Center, Faculty of Medicine, Assiut University. It included 50 subjects; 40 naïve breast cancer patients and 10 age matched apparently healthy individuals as controls. Patients were selected based on the following criteria: naïve breast cancer patients without any therapeutic interposition classified from stage I to stage IV according to the National Comprehensive Cancer Network (NCCN) clinical practice guidelines in oncology.[14] Breast cancer patients with previous chemotherapy or radiotherapy treatment were excluded. Tumor estrogen/progesterone receptor (ER/PR) status and HER2 status were determined by immunohistochemistry (IHC). Demographic data, clinicopathological characteristics, and reproductive history at the time of presentation were collected and evaluated. Such data are routinely recorded on the patient's file sheet questionnaire by the examining physician including age, marital status in addition to family history of breast cancer. Data on tumor size and nodal status were obtained by examination of the tissue biopsies. Abdominal ultrasound and chest X-rays, CT chest and abdomen and bone scan were carried out to exclude metastases. The control criteria included age matched women with no signs or symptoms of breast cancer. Women with chronic infections, autoimmune diseases, or who receive immunosuppressive treatment were not included in the control group.

Peripheral Blood Mononuclear Cell (PBMCs) Isolation

Five milliliters of blood were withdrawn from each subject under complete aseptic conditions into heparinized tubes. Peripheral blood mononuclear cells (PBMCs) were isolated based on density-gradient centrifugation using Ficoll-Paque (Histopaque-1077, Sigma-Aldrich).[15]

Analysis of B-regs surface markers, induction and detection of intracellular cytokine (IL-10) by Flow cytometry

PBMCs were re-suspend in 1mL of warm RPMI media, add 2µl of cell stimulation cocktail (500X); phorbol 12-myristate 13-acetate (PMA) (100 ng/mL, Sigma-Aldrich) and ionomycin (1µg/mL, Sigma-Aldrich). One microliter of Brefeldin A (1000X) (BioLegand, USA), a protein transport inhibitor, was added to block cytokine secretion at the Golgi. The cells were then washed with PBS, and Fc receptors were blocked using 10µL of human serum and incubated for 15 min at room temperature in a dark place. After incubation, an extracellular staining was performed by incubating the cells with fluorescently labeled antibodies (Table 1) for 30 minutes at room temperature in a dark place. Cells were then fixed and permeabilized using Flow Cytometry Fixation / Permeabilization Buffer I (1X, R&D systems, Bio-

Techne, USA), stained with anti-IL-10 (Table 1) and the mixture incubated for 30-45 minutes at 2-8 °C. Cells were then washed and resuspended in PBS, and measured by FACS Caliber (Becton Dickinson, USA) flow cytometer.[16] An isotype-matched negative control was used for each sample. Analysis was performed using FlowJo (Version 10) software.

Statistical analysis

All statistical analyses were carried out using SPSS for Windows version 17 (SPSS, Chicago, IL, USA). Graphs were created in Prism 7 (GraphPad Software Inc., San Diego, CA, USA). Quantitative data were expressed as mean ± standard deviation. The statistical significance was assessed with unpaired two-tailed Student's t test and one-way ANOVA test. Two-sided p-values were calculated; the differences were considered statistically significant at $P < 0.05$.

Table 1. List of used antibodies

Antigenic Target	Fluorochrome	Clone	Manufacturer
CD19	PerCP	4G7-2E3	R&D systems
CD24	APC	ML5	R&D systems
CD27	FITC	LG.7F9	e-Bioscience
IL-10	PE	REA1008	Miltenyi Biotec

Results

Clinical and pathological data of patients

Table 2 shows that 33 (82.5%) and 7 (17.5%) patients were diagnosed as invasive ductal carcinoma (IDC), and invasive lobular carcinoma (ILC), respectively. BC laterality was not associated with human histology (P=0.7931). According to the NCCN guidelines in oncology of 2018; 1 of 40 (2.5%) was in stage I, and 37 of 40 (92.5%) were in stage II, 1 of 40 (2.5%) was in stage III, and 1 of 40 (2.5%) in stage IV. The estrogen receptor positive (ER+)/progesterone receptor positive (PR+) phenotype was recorded in 75% (30/40) of the studied population while the ER-/PR- variant were displayed in 17.5% (7/40) of them. BC laterality was not associated with ER/PR or Her-2 expression (P> 0.05 for both). Also, BC laterality was not associated with TNM or TNM stage grouping (P> 0.05). Tumor metastasis in all cases was

undetermined. The risk factors for breast cancer are shown in Table 3.

Analysis of regulatory B cells in the studied groups

Frequency of CD19+ B cells in peripheral blood of patients and controls

The mean percentage of CD19+ B cells in BC patients represented 13.46% of the cells in patients' lymphocyte gate compared to control individuals with mean percentage of 16.48% (Figure 1: A, B1 & B2). There was a significant decrease in CD19+ B cells percentage in patients compared to controls ($P < 0.0001$).

Frequency of CD19+IL10+ B cells (B-regs) in peripheral blood of patients and controls

The frequency of B10 cells (CD19+IL10+ B cells) in BC patients was 4.82% of B cells which was significantly higher than B10 cells in control individuals ($P < 0.0001$) with a percentage of 1.05% of B cells (Figure 1: C1, C2).

Frequency of CD19$^+$CD24^{hi}CD27$^+$, and CD19$^+$CD24^{hi}CD27$^+$IL10$^+$ B-regs phenotypes in peripheral blood of patients and controls

As B-regs have different phenotypes, we analyzed cell surface markers as CD24, and CD27 after *in vitro* stimulation to identify different phenotypes of B-regs. In BC patients, CD19$^+$CD24^{hi}CD27$^+$ B cells composed 13.82 % of B cells which was significantly lower compared to control individuals ($P < 0.0001$), which composed 19.02% of B cells (Figure 1, D1& D2). Also, it was found that CD19$^+$CD24^{hi}CD27$^+$IL10$^+$ B cells composed 1.75% of total B cells in BC patients, significantly higher than these detected in in control individuals ($P < 0.0001$) with a percentage of 0.64 % of total B cells (Figure 1, E1 & E2).

Table 2. Clinical and pathological data of patients.

Characteristic	Total BCs	Left BCs		Right BCs		P-value	LRR
	N	N	%	N	%		
*Histology: (n=40)							
Ductal	33	18	54.5	15	45.56		1.2
Lobular	7	5	71.4	2	28.6	NS	2.5
Mixed ductal and lobular	0	0	0.0	0	0.0		0
*Grade: (n=40)							
1	1	1	100	0	0.0		_
2	37	20	54.05	17	45.95	NS	1.18
3	1	1	100	0	0.0		_
4	1	1	100	0	0.0		_
*Hormonal Receptors:							
ER+/PR+	30	17	56.67	13	43.33		1.31
ER+/ PR-	2	1	50	1	50	NS	1
ER-/ PR+	1	1	100	0	0		-
ER-/ PR-	7	4	57.14	3	42.86		1.33
*HER2: (n=21)							
Non over expressed (0-2+)	19	10	52.6	9	47.4	NS	1.1
Over expressed (3+)	2	2	100	0	0.0		_
*T stage: (n=40)							
1	6	4	66.67	2	33.33		2
2	21	11	52.38	10	47.62	NS	1.1
3	8	4	50	4	50		1
4	5	4	80	1	20		4
*N stage: (n=40)							
1	20	11	55	9	45		1.22
2	12	7	58.3	5	41.67	NS	1.4
3	8	5	62.5	3	37.5		1.67
M0 stage	40	-	-	-	-		-
*TNM stage group:							
II	17	10	58.82	7	41.18	NS	1.43
III	23	13	56.5	10	43.5		1.3

ER: estrogen receptor, PR: progesterone receptor, HER2: human epidermal growth factor receptor, TNM: tumor, node, and metastasis., $P > 0.05$ is not significant (NS).

Table 3. Breast cancer associated risk factors.

Risk factor	Cases (*N*=40)		Controls (*N*=10)		Odds ratio (95% CI)	*P*-value
	No.	%	No.	%		
Age of marriage:	(N=37)		(N=9)			
* more than 21 years	20	54.05	3	33.33	2.353	
* Up to 21 years	17	45.95	6	66.67	(0.5754- 9.437)	NS
Age of 1st pregnancy (years):	(N=33)		(N=8)			
*≥ 25years	14	42.42	3	37.5	1.228	
*< 25 years	19	57.58	5	62.5	(0.2426-5.226)	NS
Age at last childbirth (years):	(N=33)		(N=8)			
*Up to 28	10	30.3	2	25	1.304	
*More than 28	23	69.7	6	75	(0.236 - 7.18)	NS
Total breastfeeding duration:	(N=33)		(N=8)			
*Up to six months	7	21.21	1	12.5	1.885	
*More than six months	26	78.79	7	87.5	(0.2124- 23.96)	NS
History of abortions:	(N=37)		(N=9)			
*Yes	11	29.73	2	22.22	1.481	
*No	26	70.27	7	77.78	(0.3068 -7.88)	NS
History of oral contraceptive pills:	(N=37)		(N=9)			
*Yes	16	43.24	2	22.22	2.667	
*No	21	56.76	7	77.78	(0.5814 -13.79)	NS
Age at menopause (years):	(N=22)		(N=3)			
*More than 50	14	63.64	2	66.67	0.875	
*Up to 50	8	36.36	1	33.33	(0.05427-8.503)	NS
Family history of breast cancer:						
*Yes	21	52.5	2	20	4.421	
*No	19	47.5	8	80	(0.8211-22.12)	NS

N: number, %: percentage, CI: confidence interval. *P*> 0.05 is not significant (NS).
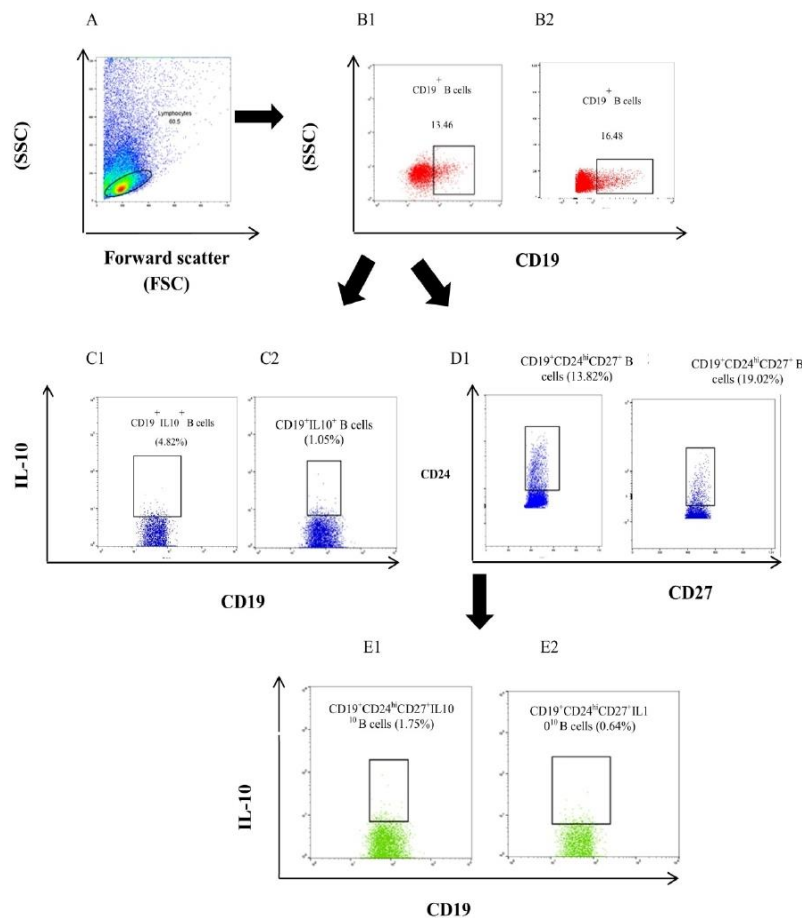
Figure 1. Representative gating strategy for identifying B-cell subsets. (A) lymphocytes are gated on the forward scatter (FSC)/side scatter (SSC) dot plot of PBMCs population. (B) CD19$^+$ B cells a frequency in PBMCs from BC patients and controls; (B1) represents CD19$^+$cells were gated on side scatter and CD19 dot plot in BC patient. (B2) CD19$^+$cells were gated on side scatter and CD19 dot plot in healthy control. (C) Frequencies of CD19$^+$IL10$^+$ B cells in healthy individuals and BC patients after *in vitro* stimulation; (C1) Dot plotting represents B10 cells frequency in BC patient, (C2) Dot plotting represents frequency of B10 cells in healthy individual. (D) Frequencies of CD19$^+$CD24^{hi}CD27$^+$ B-regs in healthy individuals and BC patients after *in vitro* stimulation; (D1) Dot plotting represents percentage of CD19$^+$CD24^{hi}CD27$^+$ B cells in total CD19$^+$ B cells from BC patient, (D2) Dot plotting represents percentage of CD19$^+$ CD24^{hi} CD27$^+$ B cells in total CD19$^+$ B cells in healthy control. (E): Frequencies of CD19$^+$CD24^{hi}CD27$^+$IL10$^+$B-regs phenotype in the blood of BC patients and healthy individuals after *in vitro* stimulation; (E1) Dot plotting represents percentage of CD19$^+$CD24^{hi}CD27$^+$IL10$^+$B cells detected in BC patient, (E2) Dot plotting represents percentage of CD19$^+$ CD24^{hi}CD27$^+$IL10$^+$B cells detected in healthy control.

The relation between circulating B-regs phenotypes and different clinical features of BC patients

The percentage of CD19$^+$IL10$^+$ (B10) B cells phenotype was significantly associated with the HER-2 expression levels, and T, and N stages (Figure 2: A, B, C). However, there was no significance association between this phenotype and other clinical features of BC patients (*P* > 0.05 for all). The CD19$^+$CD24^{hi}CD27$^+$ and CD19$^+$CD24 ^{hi}CD27$^+$IL10$^+$ B cells phenotypes were not significantly associated with any of the clinical features of BC patients (*P* > 0.05) as shown in Table 4. The ER/PR expression levels were not significantly associated with regulatory B cells phenotypes (Figure 3: A, B, C).
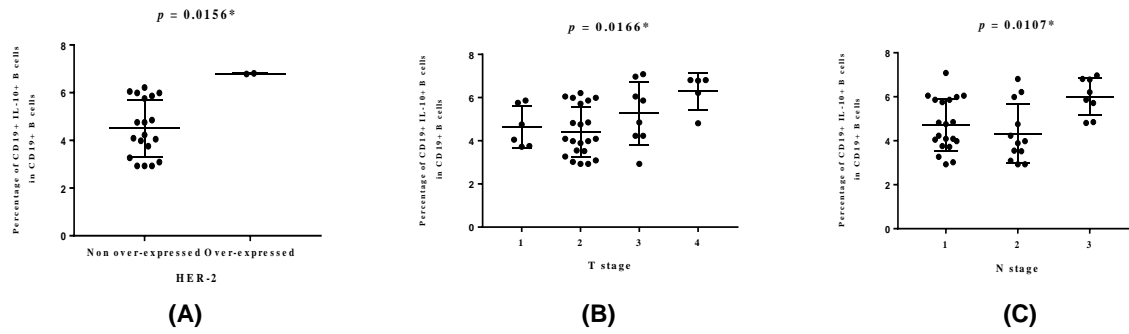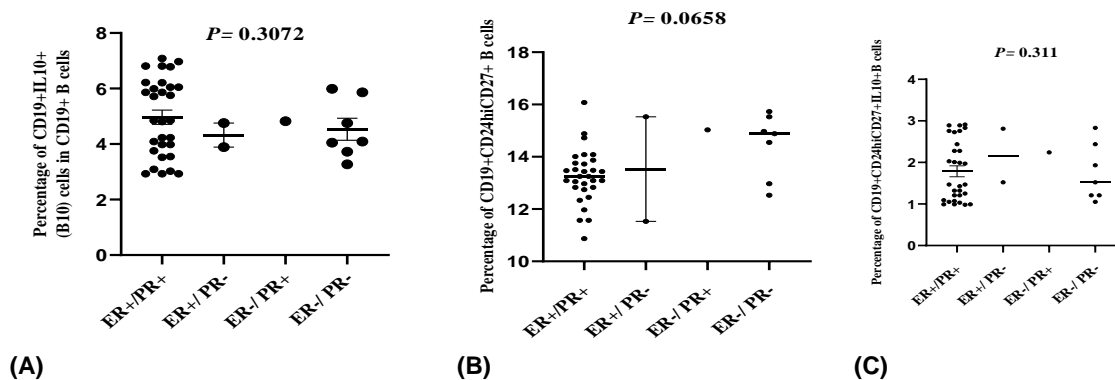
Figure 2. (A) Percentage of CD19^{+}IL10^{+} (B10) cells in CD19^{+} B cells in BC patients with different HER-2 expression levels. (B) Percentage of CD19^{+}IL10^{+} (B10) cells in CD19^{+} B cells in BC patients at different T stages. (C) Percentage of CD19^{+}IL10^{+} (B10) cells in CD19^{+} B cells in BC patients at different N stages. Data are expressed as mean ±SD.

Table 4. Association between circulating B-regs phenotypes and clinico-pathological characteristics of BC patients.

Characteristics	Number of cases	CD19^{+}IL10^{+} (B10) cells	*P-value	CD19^{+}CD2^{hi}CD27^{+} B cells	P-value	CD19^{+}CD2^{hi}CD27^{+}IL10^{+}B cells	P-value
*Age:							
25-44	11	5.215±0.39		13.95±0.45		1.634±0.19	
45-64	23	4.632±0.26	NS	13.53±0.23	NS	1.779±0.15	NS
≥ 65	6	5.038±0.64		12.69±0.33		2.53±0.30	
*Histology:							
Ductal	33	4.747 ± 0.23	NS	13.43± 0.22	NS	1.864 ± 0.13	NS
Lobular	7	5.353 ± 0.43		13.92 ± 0.42		1.557 ± 0.19	
*Grade:							
1	1	4.05±0.0		14.55±0.0		1.93±0.0	
2	37	4.77±0.21	NS	13.49±0.21	NS	1.826±0.12	NS
3	1	6.81±0.0		13.25±0.0		1.47±0.0	
4	1	6.78±0.0		13.87±0.0		1.43±0.0	
*Hormonal Receptors:							
ER+/PR+	30	6.71±0.26		13.25±0.19		1.79±0.13	
ER+/ PR-	2	4.33±0.44	NS	13.53±0.20	NS	2.17±0.65	NS
ER-/ PR+	1	4.82±0.00		15.03±0.00		2.24±0.00	
ER-/ PR-	7	4.53±0.40		14.45±0.47		1.74±0.26	
*HER-2: (*n*=21)							
Non over expressed (0-2+)	19	4.5 ± 0.27	0.0156	13.82 ± 0.31	NS	1.691±0.17	NS
Over expressed (3+)	2	6.795 ± 0.2		13.56 ± 0.31		1.45±0.02	
*T stage:							
1	6	4.652±0.97		13.83±0.74		1.548±0.22	
2	21	4.409±0.25	0.0166	13.64±0.24	NS	1.892±0.17	NS
3	8	5.275±0.52		12.94±0.34		2.006±0.27	
4	5	6.286±0.39		13.57±0.13		1.464±0.36	
*N stage:							
1	20	4.711±0.26		13.93±0.28		1.827±0.16	
2	12	4.325±0.39	0.0107	13.07±0.34	NS	1.803±0.23	NS
3	8	6.001±0.30		13.17±0.32		1.779±0.20	
*TNM stage group:							
I + II	17	4.628±0.31	NS	13.58±0.34	NS	1.756±0.19	NS
III	23	5.02±0.27		13.47±0.23		1.85±0.13	

ER: estrogen receptor, PR: progesterone receptor, HER2: human epidermal growth factor receptor, TNM: tumor, node, and metastasis. *P> 0.05 is not significant (NS).

Figure 3. (A) Percentage of CD19$^+$IL10$^+$ (B10) cells in CD19$^+$ B cells in BC patients with different ER/PR levels. (B) Percentage of CD19$^+$CD24^{hi}CD27$^+$ B-regs in BC patients with different ER/PR levels. (C) Percentage of CD19$^+$CD24^{hi}CD27$^+$IL10$^+$B cells in BC patients with different ER/PR levels. Data are expressed as mean ±SD.

Discussion

Breast cancer is ranked within the most common female neoplasms worldwide, which is increasing particularly in developing countries where the majority of cases are diagnosed in late stages. The peak frequency of breast cancer increased with age until menopause, thereafter it started to decline. The mean age of BC patients in this study was (51.53 ± 1.95) years; this is in accordance with Gabr et al. (2016) who reported that two third of the patients were between their forties and sixties, displaying a noticeable decline after the age of 60 years.[17] World Health Organization (WHO) estimates revealed that half of the cancers in the Eastern Mediterranean Region (EMR) occur before the age of 55 years.[18]

In this study BC involves left side (LS) more than right side (RS) with LS-to-RS ratio (LRR) of 1.35. LS breast cancer is more predominant in (55-64) age group with 20 % from total cases. The leading tumor type was IDC; 82.5% followed by ILC; 17.5%. Makar, (2019) reported that the most common histopathological type was IDC and approximately one third of the patients had grade III tumor.[19] According to the NCCN guidelines in oncology, 2018; 92.5% were in stage II. Similar results were reported by a previous study in Egypt; Gihan et al. (2019). They found that most BC cases were either stage II (37%) or stage III (44.6%) while 4.2% and 14.2% of cases in Stage I and IV, respectively.[20] Surveys from other developing countries reported high proportions of advanced stages at presentation.[21] Such finding could be explained mainly by the lack of early detection programs which results in a high proportion of women presented with late-stage disease. Furthermore, absence of adequate diagnosis and treatment facilities could lead to the same observation.

Patients with hormone receptor-expressed tumors, such as ER+ and/or PR+ will gain effective endocrine therapy which is usually offered after surgery and radiotherapy for BC. It can reduce the risk of deaths due to breast cancer and recurrence in those patients. Hormone receptor status and HER2 status are of critical interest in determining the prognosis of breast cancer patients. Their status is routinely assessed by IHC.[22] While the ER+/PR+ phenotype was recorded in 75% of our studied patients, the ER-/PR- variant 17.5%, the ER+/PR- 5% and the ER-/PR+ 2.5%. Gabr et al., (2016) reported that the ER+/PR+ phenotype was recorded in 40.6% of the studied population, the ER-/PR- variant displayed in 30.4%, and ER/PR undetermined in 20%.[17] HER2 is a growth-promoting protein on the outside of all breast cells. Breast cancer cells with higher-than-normal levels of HER-2 are called HER-2-positive. These cancers tend to grow and spread faster than other breast cancers, but are much more likely to respond to treatment with drugs that target the HER2 protein.[23] Among our Egyptian BC patients only 9.52% were HER-2-positive. BC laterality was not associated with expression of ER/PR or HER-2; however their expression was more predominant in left-sided tumors. Also, BC laterality was neither associated with TNM classification nor TNM stage grouping.

Recently, research have adapted a new wave to identify the role of B-regs which had immunosuppressive functions in carcinogenesis. B-regs, an important B cell subset producing IL-10, had been shown to contribute to autoimmune diseases, cancers, and chronic infections.[24]

This study found that that the proportion of total B cells among PBMCs was significantly higher in the controls than in BC patients with a mean percentage of 16.48 %, and 13.46 %, respectively. Tsuda et al. (2018) found differences in B-cell differentiation between BC patients and healthy donors. Low-B patients had the same proportion of B cells as the control group, while the proportion of B cells in high-B patients was higher than in the controls. Their statistical analysis revealed that B-cell increase to be due to the significantly increased proliferation or survival of memory B cells in the high-B group compared with the other groups. The increase in B cell in BC patients and the increase in memory B cells are indicative of previous humeral immunity activation in BC patients, although the cause of this and its implications for cancer progression cannot be determined.[25] This variation may be attributed to the difference between studied patient populations, the difference between the number of investigated specimens, also, the stage and subtype of B cells were not considered in that study. In future studies, it will be necessary to conduct more detailed analyses on a larger number of clinical samples.

In this study, the frequency of circulating B-regs (CD19$^+$IL10$^+$ B cells) was increased in BC patients than in controls with a mean percentage of 4.85%, and 1.16%, respectively. Although there are limited number of studies about B-regs frequency in BC, but there are studies concerning the role of B-regs in other types of cancers, which agreed with our results. In a study about lung cancer, it was found that the frequency of CD19$^+$IL10$^+$ B cells in lung cancer patients was significantly higher than in control individuals.[26] Such finding may indicate that B-regs are associated with immune evasion in BC. Another study in Egypt found that the frequency of B-regs was significantly higher in hepatitis C virus patients with hepatocellular carcinoma compared to the controls.[27] A recent study on cervical carcinoma found that B-reg cells in cervical cancer patients were significantly increased, which was closely related to disease progression.[28]

The proportion of CD19$^+$CD24^{hi}CD27$^+$ B-regs phenotype was significantly lower in BC patients than in the controls ($P < 0.0001$). This finding was close to that reported by another study which found significant decrease in CD24^{hi}CD27$^+$ B cells in metastatic lymph node (MLNs) in BC, and explained the relation between this B-regs phenotype and lymph node metastasis.[29] Another study on gastric cancer confirmed that the CD19$^+$CD24^{hi}CD27$^+$ B cell population contained cells that suppressed the proliferation of CD4$^+$ T cells and inhibited the secretion of IFN-gamma by autologous CD4$^+$ T cells, indicating that the CD19$^+$CD24^{hi}CD27$^+$ B cell population contained B-regs. However, the frequency of B-regs was not high even in the CD19$^+$CD24^{hi}CD27$^+$ B cell subset, and B-regs were also contained in other populations, indicating that this phenotyping strategy could not uniquely identify B-regs.[30]

However, the proportion of CD19$^+$CD24^{hi}CD27$^+$IL10$^+$ B-regs phenotype was significantly higher in BC patients than in controls ($P < 0.0001$). The percentage of CD19$^+$IL10$^+$ B cells phenotype was significantly associated with the HER-2 expression levels, T, and N stages of BC. However, there was no significant association between this phenotype and other clinical features of BC patients like age, BC histology, BC grade, ER/PR expression levels. For CD19$^+$CD24^{hi}CD27$^+$ and CD19$^+$CD24^{hi}CD27$^+$IL10$^+$ B cells phenotype, there were not significantly associated with any of clinic-pathological features of BC patients. This may indicate that B-regs of memory B cells origin are not related to clinical features of BC patients. Murakami et al. (2019) found that increased numbers of B-regs were significantly correlated with poor prognosis in gastric cancer patients although there was no significant correlation between the frequency of B-regs and clinicopathologic characteristics.[30]

In conclusion, high percentage of B-regs phenotypes CD19$^+$IL10$^+$ and CD19$^+$CD24^{hi}CD27$^+$IL10$^+$ in BC patients may indicate a possible role in immune-suppression during the development of BC. The percentage of CD19$^+$IL10$^+$ B cells phenotype was

significantly associated with the HER-2 expression levels, T and N stages of BC.

Author Contributions

All authors made a significant contribution to this work, whether that is in the conception, study design, acquisition of data, analysis and interpretation, or in drafting, revising or reviewing the article and gave final approval of the version to be published.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval

The study protocol was reviewed and approved by the Ethical Committee of the Faculty of Medicine, Assiut University (Approval number 17200384).

Informed consent

Informed written consent was obtained from all participants before enrolment.

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