RESEARCH ARTICLE

Prognostic significance of FOXP3+ tumour-infiltrating Tregs in Egyptian breast cancer patients

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Abstract

Objective: To evaluate the prognostic importance of tumour-infiltrating forkhead box P3 protein + regulatory T cells in breast cancer patients.

Methods: The case-control study was conducted from January 2020 to March 2021 at the Kafrelsheikh University Hospital, Egypt, and comprised individuals with newly-diagnosed breast cancer who underwent conventional surgery, and controls who had a fibrocystic change of the breast. The density of tumour-infiltrating forkhead box P3 protein + regulatory T cells was assessed by immunohistochemistry. Overall survival and disease free-survival were assessed. Data was analysed using SPSS 25.

Results: Of the 100 patients having mean age 44.9±9.1 years, 76(76%) had moderate/strong forkhead box P3 protein expression in tumour-infiltrating regulatory T cells, and 24(24%) with no/low expression. On follow-up, Patients with moderate/strong expression had a significantly greater rate of recurrence (p<0.05). Disease-free survival was substantially shorter in patients with moderate/strong expression compared to those with little or low expression (p=0.035). Compared to individuals with no/low expression, patients with moderate/strong expression had a greater rate of mortality, but the difference was not statistically significant (p>0.05).

Conclusions: High density of forkhead box P3 protein + regulatory T cells in Egyptian women with breast cancer may serve as a prognostic indicator.

Keywords: T-Lymphocytes, Prognosis, Immunohistochemistry, Breast neoplasms, X chromosome.

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Introduction

Breast cancer (BC) has a wide range of morphological characteristics and a variable clinical prognosis.[1] It is the most prevalent malignancy and the most likely reason for a woman to die from cancer anywhere in the world.[2] Unfortunately, BC rates are rising in underdeveloped countries, like Egypt. The population's aging, the postponing of the first pregnancy, the decline in breastfeeding rates, and the shift to high-calorie Western diets are mostly responsible for the spike. BC is the most prevalent cancer in women, accounting for around 32% of all female malignancies, with crude and age-standardised incidence rates of 35.8 and 48.8 per 100,000 people, respectively, according to Egypt's National Population-Based Cancer Registry Programme.[3] Also, in comparison to younger age groups, the older age group had a significantly higher score on the BC subscale.[4] When compared to the United States (14.7 per 100,000), Egyptian breast cancer patients had a higher mortality rate (20.1 per 100,000).[5]

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Immunology of BC suggests a dual role of immune cells, including tumour-suppressive or immunosuppressive effect, according to the carcinogenic environment signals.[6] Among these cells, regulatory T cells (Treg) were found in large numbers in the tumour tissues, lymph nodes (LNs) and circulation of many cancers, including BC.[7]

Forkhead box P3 (FOXP3) protein is a transcription factor from the winged helix family that is encoded on the X chromosome. Since only discrete populations of Treg cells express FOXP3 in the nucleus, it is thought to be a unique marker for identifying Treg cells in vivo.[8]

The transcription factor FOXP3 is among the best-characterised indicators for Treg cells.[9]

FOXP3 is a 47-kDa protein composed of 431 amino acids, and it belongs to the FOX protein family's subfamily P. Members of the subfamily have conserved deoxyribonucleic acid (DNA)-binding domains with 3 alpha (α) helices and 2 sizable loops that resemble the twin wings of butterflies.[10]

There is controversy about the prognostic significance of FOXP3+ Treg cells in various malignancies.[11] The importance of FOXP3+ Treg cells for BC prognosis is also highly variable. One meta-analysis found a link between poor survival and BC patients' high expression of FOXP3+

Treg cells that infiltrated the tumour.[12] In another meta-analysis, LN status, oestrogen receptor (ER) status, and progesterone receptor (PR) status were all related to FOXP3+ Treg cell expression.[13]

The current study was planned to stress the importance of FOXP3+ Treg cells that infiltrate tumours in BC patients.

Patients and Methods

The case-control study was conducted from January 2020 to March 2021 at the Kafrelsheikh University Hospital, Egypt. After approval from the institutional ethics committee, the sample was raised from among patients attending the clinics and met the inclusion criterion as a comprehensive sample.

Those included were newly-diagnosed BC patients who underwent conventional surgery. Those receiving alternative forms of treatment, including chemotherapy, radiotherapy or targeted therapy, were excluded. Besides, there were patients with fibrocystic change of the breast who served as the controls.

After taking informed consent from the participants, they were subjected to detailed history and relevant clinical examination. Tumour staging and grading were done according to the Tumour-Nodes-Metastasis (TNM) classification and World Health Organisation (WHO) recommendations.[14]

Correlation between FOXP3 and clinico-pathological parameters, like age, affected side, tumour pathology, grade, stage and hormonal status was explored.

Immunohistochemistry (IHC) for ER, PR, and human epidermal growth factor receptor-2 (HER-2/neu) was used to determine the hormonal status of the studied tumours.[15]

For IHC, sections of 4µm thickness from each case were stained for FOXP3. Each run included a negative control slide by bypassing the primary antibody. In each run, positive control slides for FOXP3 (sections from normal tonsillar tissue) were added. Streptavidin-biotin amplified system was the technique used for immunostaining, using rabbit monoclonal antibody against FOXP3, and antibody concentration was ready to use. The detector kit was an anti-polyvalent horseradish peroxidase (HRP) / 3,3'-Diaminobenzidine (DAB) detection system using Ultra Vision LP (Lab Vision, USA). Purified goat polyvalent anti-mouse immunoglobulin G (IgG), which can bind to the primary antibody, was the employed biotinylated secondary anti-immunoglobulin. Finally, the reaction was visualised by DAB reagent. FOXP3 density was subjectively assigned in tumour-infiltrating lymphocytes as mild, moderate and strong expressions.[16] Antigen retrieval step was heat-mediated (heat-induced epitope retrieval [HIER]).[17]

FOXP3 stained slides were evaluated 1a showing cytoplasmic tumour cells and 1b with nuclear tumour-infiltrating lymphocytes. FOXP3 staining status in tumour epithelial cells was negative with 0-25% of tumour cells stained by FOXP3, and positive with >25% of tumour stained by FOXP3.[18]

For lymphocytic FOXP3 expression, scores of 0 and 1+ were considered negative, whereas scores of 2+ and 3+ were considered positive.[16]

Both disease-free survival (DFS), which is measured from the time of diagnosis until recurrence or death, and overall survival (OS), which is measured from the time of diagnosis until death, were used as outcome parameters.

Data was analysed using SPSS 25. Data was expressed as mean ± standard deviation (SD) and frequencies and percentages, as appropriate. Chi-square test or Fisher's exact test was used to compare categorical variables, while t-test was used to assess numerical data. The survival was compared using Kaplan-Meier analysis with log-rank comparison. In order to find predictors of DFS, Cox-hazard regression analysis was performed, and to find predictors of OS, logistic regression analysis was used. $P<0.05$ was considered statistically significant.

Results

Of the 100 patients having mean age 44.9±9.1 years, 76(76%) had moderate/strong FOXP3 expression in tumour-infiltrating Treg cells (Figure 1A-B), and 24(24%) with no/low expression (Figure1C).

There was a significant lower frequency of women with positive Her2 in the moderate/strong expression group ($p<0.05$). On follow-up, patients with moderate/strong expression had a considerably greater recurrence rate ($p=0.039$).

Compared to FOXP3 cytoplasmic expression in the cases (Figure 1D), the controls showed nuclear expression (Figure 2) and 13(86.7%) in the control group were FOXP3–positive. The difference between the groups regarding FOXP3 expression in epithelial cells was significant ($p<0.05$).

When compared to individuals with no/low expression, patients with moderate/strong expression had considerably lower DFS ($p=0.035$) (Figure 3). Patients in the moderate/strong expression group had higher rate of mortality compared to patients with no/low expression, but the difference was not statistically significant (Table 1).

In univariate analysis, moderate/strong expression showed

Table-1: Comparison between patients with no/low and moderate/high FOXP3+ Treg cells regarding the clinic-pathological and prognostic data

	All patients N=100	**Moderate/strong FOXP3+ Tregs n=76**	**No/low FOXP3+ Tregs n=24**	***p-value***
Age (years) mean ± SD	44.9 ± 9.1	44.7 ± 8.9	45.7 ± 10.1	0.74
Affected side n (%)				
Right	44 (44.0)	32 (42.1)	12 (50.0)	0.5
Left	56 (56.0)	44 (57.9)	12 (50.0)	
Tumour pathology n (%)				
Ductal carcinoma	74 (74.0)	58 (76.3)	16 (66.7)	0.64
Lobular carcinoma	10 (10.0)	6 (7.9)	4 (16.7)	
Medullary carcinoma	6 (60.0)	4 (5.3)	2 (8.3)	
Mucinous carcinoma	8(8.0)	6 (7.9)	2 (8.3)	
Papillary carcinoma	2 (2.0)	2 (2.6)	-	
Tumour grade n (%)				
2	82 (82.0)	60 (78.9)	22 (91.7)	0.16
3	18 (18.0)	16 (21.1)	12 (8.3)	
Tumour stage n (%)				
IA	28 (28.0)	22 (28.9)	6 (25.0)	0.2
IIA	58 (58.0)	46 (60.5)	12 (50.0)	
IIB	14 (14.0)	8 (10.5)	6 (25.0)	
Positive ER n (%)	72 (72.0)	56 (73.7)	16 (66.7)	0.50
Positive Her2 n (%)	2 (2.0)	-	2 (8.3)	0.011
Positive PR n (%)	72 (72.0)	56 (73.7)	16 (66.7)	0.5
Triple-negative n (%)	26 (26.0)	20 (26.3)	6 (25.0)	0.9
Recurrence n (%)	24 (24.0)	20 (26.3)	4 (16.7)	0.039
DFS (months) mean (95% CI)	36.0 (33.9-38.1)	34.7 (32.1-37.3)	39.8 (39.2-40.4)	0.035
Mortality n (%)	10 (10.0)	10 (13.2)	-	0.061

FOXP3: Forkhead box P3 protein, Treg: Regulatory T cells, SD: Standard deviation. ER: Oestrogen receptor, Her2: Human epidermal growth factor receptor-2, DFS: Disease-free survival, CI: Confidence interval.

Table-2: Predictors of DFS.

	Univariate analysis		
	HR	**95% CI**	***p-value***
Age	0.99	0.95-1.01	0.85
Tumour pathology			
Other pathologies	Ref	-	-
Ductal carcinoma	1.72	0.58-5.07	0.32
Tumour grade			
2	Ref	-	-
3	1.16	0.39-3.44	0.78
Tumour stage			
IA	Ref.	-	-
IIA	1.14	0.43-2.98	0.79
IIB	1.26	0.35-4.5	0.72
FOXP3 Treg cells	4.18	0.98-17.8	0.053

DFS: Disease-free survival, HR: Hazard ratio, CI: Confidence interval, FOXP3: Forkhead box P3 protein, Treg: Regulatory T cells,.

Table-3: Predictors of mortality.

	Univariate analysis		
	HR	**95% CI**	***p-value***
Age	0.95	0.87-1.03	0.2
Tumour pathology			
Ductal carcinoma Other pathologies	Ref	-	-
Ductal carcinoma	1.46	0.29-7.33	0.65
Tumour grade			
2	Ref	-	-
3	1.16	0.22-5.97	0.86
Tumour stage			
IA	Ref.	-	-
IIA	1.5	0.28-7.95	0.63
IIB	2.17	0.27-17.27	0.47

OR: Odds ratio, CI: Confidence interval.

marginal significance as a predictor of DFS (p=0.053) (Table 2). None of the studied variables could significantly predict OS (Table 3).

Discussion

FOXP3 gene mutation may lead to cancer development, which may be linked to aberrant immunological responses. The prognosis may be correlated with Treg cell infiltration into tumour cells. In order to create new therapies, it may be essential to comprehend the biology of the FOXP3 gene.[19]

Due to their immunosuppressive properties, FOXP3+ Tregs are abundant in tumour infiltrates, which is associated with a poor clinical prognosis. Increased infiltration of FOXP3+ lymphocytes in the tumour microenvironment has been linked to a poor prognosis for cancer patients in a number of earlier studies.[8,11,12] However, contrary findings have also been presented.[16]

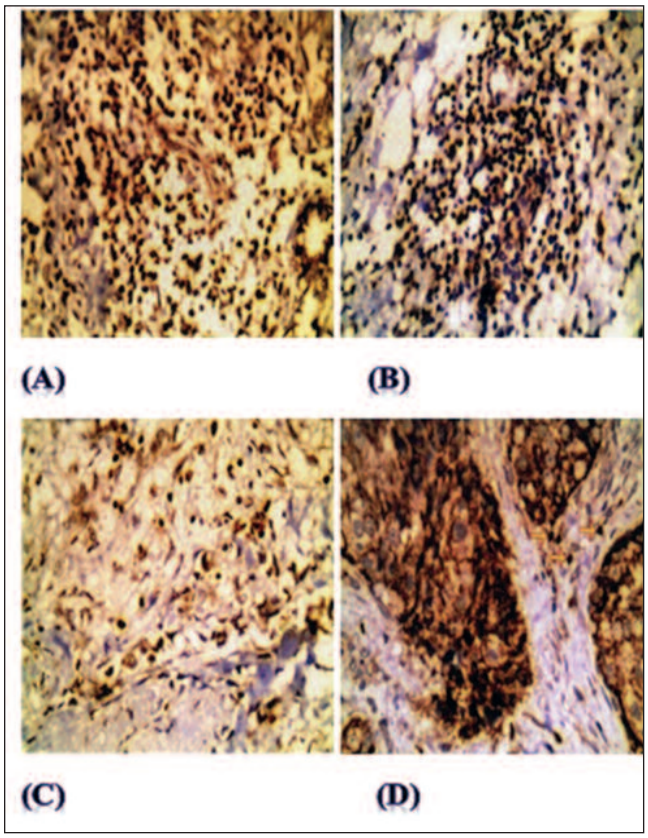

Figure 1: (A) IDC with a large number and high expression of nuclear FOXP3 + tumor-infiltrating Treg cells. (B) IDC showing moderate number and expression of nuclear FOXP3 + Treg cells. (C) IDC showing few numbers and low expression of nuclear FOXP3 + Treg cells. (D) IDC grade II showing strong granular cytoplasmic FOXP3 expression in tumour cells with a few surrounding tumour-infiltrating lymphocytes showing strong nuclear staining "arrows" (X 400).
IDC: Invasive ductal carcinoma, FOXP3: Forkhead box P3 protein, Treg: Regulatory T cells.

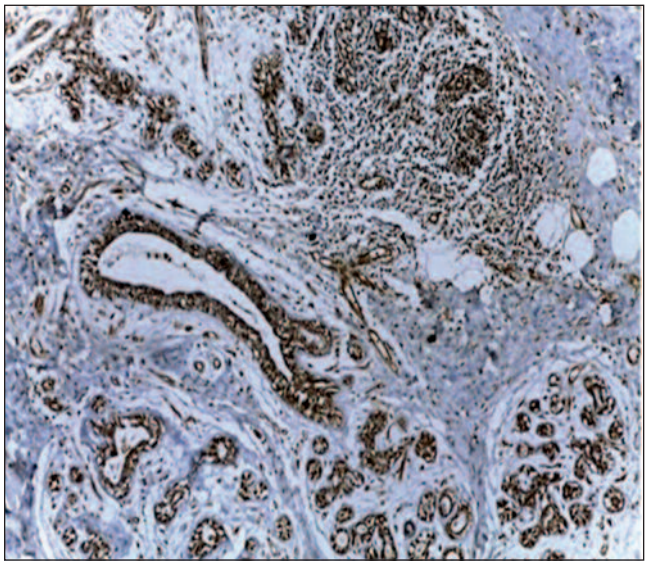

Figure 2: A case of fibrocystic change of the breast showing moderate nuclear forkhead box P3 (FOXP3) protein expression in the ductal epithelial cells as well as in lymphocytes as a control (× 100).

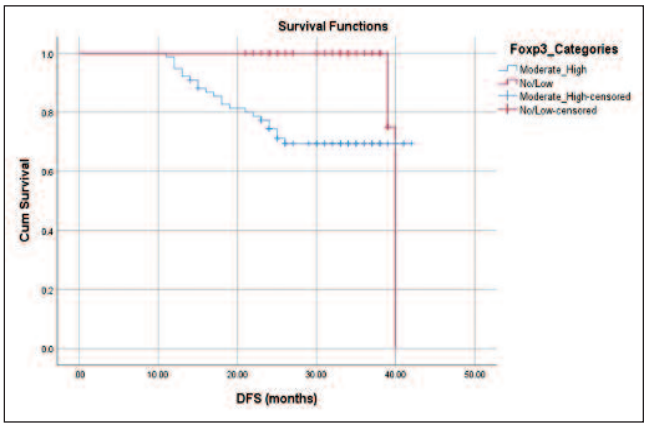

Figure 3: Disease-free survival (DFS) in the study population.

In the current study, 76% of the studied BC cases showed moderate/strong FOXP3 expression in tumour-infiltrating Treg cells. On follow-up, when compared to patients with no/low expression, patients with moderate/strong expression had a considerably greater recurrence rate and significantly shorter DFS. Moreover, patients in the moderate/strong expression group had higher rate of disease-related mortality compared to patients with no/low expression. However, the difference was not statistically significant.

The negative prognostic value of tumour-infiltrating Treg cells was previously highlighted by meta-analyses.[11,13,20] More recent studies reported similar conclusions.[21-23] Interestingly, a study in 2019[22] found an association between the existence of B regulatory and Treg cells, and associated both populations with poor prognosis in BC patients.

Controversially, in patients with triple-negative BC, higher densities of tumour-infiltrating Treg cells are related to a favourable prognostic outcome.[23] These findings may shed light on the inter-relations between the immune functions of immune-regulating cells and the hormonal receptor status with the tumour microenvironment.

A study in 2017[24] suggested that a mechanism for the immune evasion of tumour cells is triggered by the expression of FOXP3 in tumours. Signal transducer and activator of transcription-3 (STAT3), for example, may facilitate interaction between tumour cells and their immune microenvironment through FOXP3, which could result in tumour-induced immunosuppression, including the activation of Treg cells.

Targeting Tregs is a major topic since they play a crucial role in the growth of tumours. Tregs depletion is a potential strategy to encourage antitumour immunity and tumour regression.[20, 25, 26]

The current study did not calculate sample size using any scientific formula, and this can influence the power of the study, which is a limitation.

Conclusion

In Egyptian women with BC, high density of FOXP3+ Treg cells could have predictive value.

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Conflict of Interest: None.

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