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EXPRESSION OF TUMOR NECROSIS FACTOR-ALPHA (TNF- α) IN PRIMARY BREAST CARCINOMA IN IRAQI WOMEN

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ABSTRACT
Tumor necrosis factor alpha (TNF-α) is a multifunctional member of the TNF/TNFR cytokine family. It plays important role in inflammation, immunity and apoptosis, and In regard to cancer, TNF-α involves in the inflammatory network that contributes to all stages of the malignant process, stimulates cancer cells' growth, proliferation, invasion and metastasis. As well TNF-α could be a target for cancer therapy. **Methodology**: The aim of this study was to investigate the immunohistochemical (IHC) profiles of TNF-α of primary breast carcinomas in Iraqi women; And to assess the correlation of TNF-α expression and clinicopathological parameters including tumor stage, grade and lymph node metastasis. **Results:** Of the 60 carcinomas studied, 88.33% were positive for TNF-α by IHC (P=<0.05). There was no significant correlation between TNF-α expression and tumor stage, grade, or nodal involvement. **Conclusion:** TNF-α protein is strongly expressed in breast cancer and that positive expression of TNF-α may be independent from the grade and stage of tumor.

KEYWORDS: *Tumor necrosis factor alpha, cytokines, Breast cancer, Immunohistochemistry.*

INTRODUCTION
Breast cancer is the most common cancer in women worldwide in the developed and developing world, and second most common cancer overall. It represents 25% of all cancers in women and it is the fifth most common cause of cancer death in women.

Although breast cancer is thought to be a disease of the developed world, about 50% of breast cancer cases and 58% of deaths occur in developing countries.[1,2]

In Arab countries cancer registry data revealed that the lowest incidence are found in Saudi Arabia (below 12 per 100,000) and the highest in Bahrain (46.8 per 100,000).[3]

Incidence of breast cancer in Iraq, increased from 30 to 40/100,000 women between 2006 and 2012. The Iraqi Cancer Registry (2012), reported (4,115 cases) of breast cancer in year 2012, (19.5% of the newly diagnosed malignancies), with an incidence of 22/100,000 female population.[4,5]

The risk of breast cancer doubles each decade until menopause, after which the rate slows. Still breast cancer is more common after menopause.[1] Survival rates for breast cancer have improved, but not in developing countries, where it is still low due to the lack of early detection programmes, the lack of adequate diagnosis and treatment facilities, and the majority of cases are diagnosed in late stages.[6]

Inflammation plays a major role in the process of tumor genesis, and the inflammatory microenvironment is essential at different stages of tumor development. A great number of cytokines are present in tumor microenvironment and induce tumor development. One cytokine commonly found in the tumor microenvironment is TNF-α. Studies show that TNF-α is one of the pro-inflammatory cytokines with contradictory role in tumor process. It has anti-tumor properties, induces cancer cell apoptosis due to the sustained JNK (c-*jun*–NH2–kinases) activation, and as well, it has tumorigenic properties, linking inflammation with carcinogenesis through regulation of expression of cytokines promotes cell proliferation and tumor growth [7, 8] In breast tumors, pro-inflammatory cytokines, (Tumor Necrosis Factor-Alpha (TNF-a) and Interferon gamma (IFN-g)), that are secreted by tumor-infiltrating lymphocytes (TILs) play major roles in the immune response against cancer cells. Thus, TNF-α induces cancer cell death as well as, can promote tumor cell

survival, proliferation, migration and angiogenesis. It is therefore important to find out how to selectively inhibit the tumorigenic properties of TNF-α, and saving its anti-tumor properties.[9,10,11,12]

The first published data that linked TNF-α to cancer reported that treatment with TNF-α induced production of more TNF-α in breast cancer cell lines.[13] This was followed by many studies which revealed that the TNF-α secreted by inflammatory cells during tumor growth, and by tumor cells, and that TNF-α has a positive role in pro-tumerigenic process.[14] TNF-α is expressed within the tumor microenvironment, and is not normally assess in the serum of healthy persons, but increased levels have been evaluated in patients with prostate cancer, pancreatic cancer, renal cell carcinoma, hematopoietic and metastatic breast cancers.[15] It has been reported that serum TNF-α levels are approximately 20–25% higher in breast cancer tissue as compared to normal tissue and it has been suggested as a potential biomarker for prostate cancer as it is significantly elevated in tumor tissue than in normal tissue.[9] Some consider TNF-α, a possible target for cancer therapy. Larkin and co workers (2010) found that, using Infliximab, which inhibit binding of TNF-α to its receptors, does not improve clinical outcome in renal cell carcinoma.[16]

In vitro study, (using flow cytometry analyses), revealed that incubating peripheral blood mononuclear cells (PBMCs) with MCF7(a breast cancer cell line) in the presence of IL-2,rises the numbers of TNF- α secreting CD8+ T cells up to 15 times higher than PBMCs controls. However, when IL-2 was not added, the numbers of TNF- α secreting CD8+ T cells were down to 10 times lower than controls. These results suggest a vital role of IL-2 in the activation of PBMCs to secrete TNF- α against MCF7 cells. Purification of TNF- α secreting, CD45+, CD8+ and CD8-, PBMCs, and re-incubating them with MCF7 cells led to killing of MCF7 cells.[6,8]

Natural medicinal products (Withaferin A (WA) and Celastrol (Cel), have anti-cancer and anti-inflammatory effects due to more than one mechanisms, like apoptosis induction by inhibition of proteasomal activities. In accordance with the results of Li Lu and co-workers (2014), TNF-α sensitizes human breast cancer cells, MDA-MB-231 to low doses of WA and Cel, leading to apoptosis due to suppression of the nuclear translocation of nuclear factor-κB (NF-κB) signaling pathway. Thus, the anti-cancer activities of TNF-α are enhanced when combined with the natural proteasome inhibitors, WA or Cel. These results underscore the potential of natural proteasome inhibitors as a therapy in certain types of cancer, such as breast cancer.[10,11] Collectively, these data demonstrate the complexity of TNF-α in cancer pathogenesis.

Based on the essential requirement for an inflammatory microenvironment in tumor formation, we investigated the expression of TNF-α in primary breast carcinoma in Iraqi women and its correlation with different clinicopathologic features.

MATERIALS AND METHODS

Approval for use of human subjects was obtained by permission of Al Karkh- Health Directorate\Ministry of Health\ Baghdad\Iraq. Seventy-five female patients with primary breast tumors, which were diagnosed by biopsy (surgical resection specimen), at Al-Yarmouk Teaching Hospital – Histopathology unit, were identified through retrospective review of paraffin blocks and surgical pathology report databases. Patients without surgical pathology reports were excluded. Through review of surgical pathology reports, clinical information including patient age, tumor size, histologic grade, Lymph node metastases, and pathological stage, was obtained for each patient. All surgical specimens had been re-evaluated by faculty surgical pathologists at College of Medicine - Aliraqia University and specialist pathologists at Al-Yarmok Teaching Hospital. Tumors were graded according to (Scarff Bloom Richardson) combines nuclear grade, tubule formation, and mitotic rate, with 1 being well-differentiated, and 3 being poorly differentiated. For included patients, TNF–α IHC was performed on formalin-fixed, paraffin-embedded tissue sections, according to the manufacturer's instructions. Each block was cut at a thickness of 5µm on a microtome cutter (Leica RM2235). Sections were placed on salinized coated slides, (DAKO, UK) and heated at 58°C for 24 hours. Antigen retrieval step was performed by antigen retrieval solution (ready to use) using pressure cooker and scientific microwave for 20 minutes. All slides were incubated in peroxidase – blocking solution for 10 minutes, Followed by washing with DW. Slides were incubated with primary Anti-TNF alpha antibody [Mouse monoclonal, abcam (52B83)], diluted (1/100) by Ab diluent for 8 hours at 4°C. After that, sections are washed with Tris-buffered saline (TBS) and incubated with Biotinylated Link Antibody (ready to use) for 15 min. at room temperature followed by 3 washing. Then, sections were incubated with streptavidin for 15min, and then washed 3 times in wash buffer. Sections were incubated with diaminobenzidine (DAB) peroxidase for 10 min. and then washed 3times with TBS. The slides are then counterstained in hematoxylin, and finally coverslipped. Slides were scanned and scored by consultant pathologist. Positive controls of known positive tissues (liver tissue sections) and negative controls with primary antibody replaced with TBS were run with the patient slides.

Scoring of TNF alpha expression

The stained slides were quantified visually via light microscopy by two pathologists. TNF- α was brown cytoplasmic staining in invasive tumor cells. Results were determined based on the maximum staining intensity and percentage of positive tumor cells according to (Long Zhou et al., 2014), as follows; 1+ = positive cell rate <25%; 2+ = staining in at least 25–50%

of tumor cells; 3+ = strong, staining in 51–75% of invasive carcinoma cells; 4+ = staining in >75% of tumor cells.[17]

Statistical Analysis

Analysis of data was carried out using the available statistical package of SPSS-24 (Statistical Packages for Social Sciences- version 24). Overall agreement as well as score confidence intervals (CI) between the the two pathologists TNF – a IHC results were calculated. In addition, simple kappa coefficients were calculated to describe agreement. Data were presented in simple measures of frequency, percentage, mean, standard deviation, and range (minimum-maximum values). The significance of difference of different percentages (qualitative data) was tested using Pearson Chi-square test (χ2-test) with application of Yate's correction or Fisher Exact test whenever applicable. Significance was defined at $p< 0.05$.

RESULTS

Clinicopathological Parameters

The final sample size after exclusion of patients was 75 cases. All patients were females, Of the 75 cases studied, 60 were invasive ductal carcinoma, and 15 were benign fibroadenoma. The mean age of patients with invasive ductal carcinoma was 48.7 years (range 27 - 85 years), and the majority (63.33%) (38/60), were in the 5th and 6th decades. Patients with benign fibroadenoma, their mean age were 26.6 years (range 18 – 40 years) (Table – 1).The mean size of carcinomas was 3.7 cm (range 1.5 - 7cm). In terms of histologic grade, 21.70% of studied tumors were grade III (13/60), 60.00% were grade II (36/60), 18.3% were grade I (11/60). Sixty three percent of tumors (38/60) had metastasized to axillary lymph nodes, and 36.7% of patients (22/60) free of lymph node involvement. Of the 60 cases invasive ductal carcinoma, 13.3% (8/60) were stage I, 16.7% (10/60) were stage IIA, 20.0% (12/60) were IIB, 38.3% (23/60) were IIIA and 11.7% (7/60) were IIIB (table – 2).

Table 1: Age Distribution in Benign and Malignant Breast Tumors.

Age	Number of IDC - (%)	Number of Fibroadenoma - (%)
<20	-	1 (6.66)
20---29	3 (5.00)	10 (66.66)
30---39	7 (11.66)	3 (20.00)
40---49	23 (38.33)	1 (6.66)
50---59	15 (25.00)	-
60---69	10 (16.66)	-
=>70	2 (3.33)	-
Mean ± SD	48.7±11.9	26.6±5.9
Range	27-85	18-40
P value	0.0001*	
*Significant association using Pearson Chi-square test at 0.05 level.		

Table 2: Clinicopathological Parameters of 60 cases with infiltrating ductal carcinoma.

		Number of IDC - (%)
Grade	Grade I	11 (18.33)
	Grade II	36 (60.00)
	Grade III	13 (21.66)
Pathological stage	T1N0	8 (13.33)
	T2N0	10 (16.66)
	T2N1	11 (18.33)
	T2N2	11 (18.33)
	T3N0	1 (1.66)
	T3N1	5 (8.33)
	T3N2	7 (11.66)
	T4N0	3 (5.00)
	T4N1	4 (6.66)
Pathological stage	I	8 (13.33)
	IIA	10 (16.66)
	IIB	12 (20.00)
	IIIA	23 (38.33)
	IIIB	7 (11.66)
Tumor size (cm)	=<2 cm	8 (13.33)
	>2cm to 5cm	36 (60.00)
	> 5cm	16 (26.66)
Lymph Node status	Positive	38 (63.33)
	Negative	22 (36.66)

Immunohistochemical profiles

Of the 60 malignant cases, 53 (88.3%) were positive for TNF- a, and most (68.4%) (41/60) were strong positive (score 3&4), but only one benign case (6.7%) was strong positive (score 3), and other six benign (40.0%) were weak positive (score1). A **statistically significant expression** of TNF-α in malignant tumors was identified (p=0.05).). Data and morphology of Immunohistochemical staining is depicted in Table 3 and Figure 1.

Table 3: **Immunohistochemical Data and TNF-α Expression in Benign and Malignant Breast Tumors.**

TNF-alpha Expression Score		Number of IDC - (%)	Number of Fibroadenoma - (%)
TNF-alpha Expression	0 (Negative)	7 (11.66)	8 (53.3)
	1	3 (5.00)	6 (40.0)
	2	9 (15.00)	-
	3	22 (36.66)	1 (6.7)
	4	19 (31.66)	-
	P value	0.0001*	
*Significant association using Pearson Chi-square test at 0.05 level.			

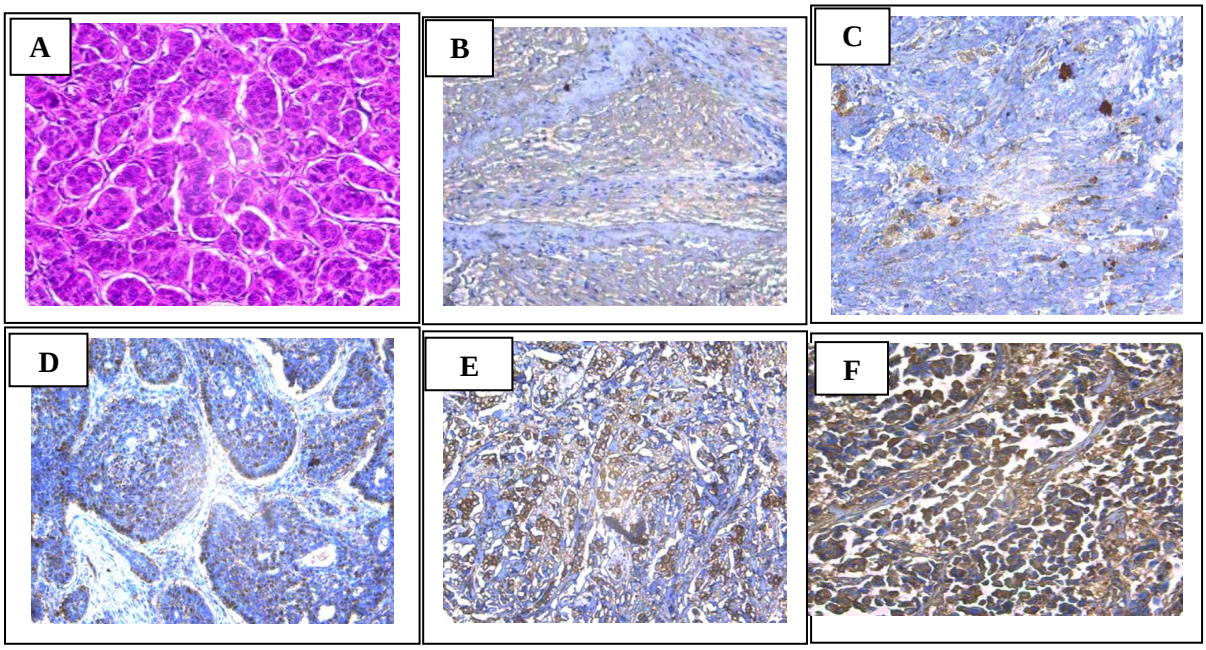

Figure 1: A. Infltartive Ductal Carcinoma (IDC), Grade II (40x, H&E), (B-F); TNF-*α* immunohistochemical expression in tumor cells (brown cytoplasmic stain). B. TNF-*α* positive expression in benign fibroadenoma sections, weak staining (score 1+, weak < 25%) (40x). C. TNF-*α* positive expression in IDC (score 2+, moderate, 25%) (40x), D. TNF-*α* positive expression in IDC (Score 3+, strong, 51%) (40x), E. TNF-*α* positive expression in IDC (Score 3+, strong, 75%)) (40x), F. TNF-*α* positive expression in IDC (Score 4+ Strong, > 75%) (40x).

The Correlation of the expression of TNF- α with the clinicopathologic features

Expression of TNF-α showed a slight increase in malignant tumors with higher grade, higher stage larger size, and positive lymph node metastases compared with that of lower grade, lower stage smaller size and negative lymph node involvement. This positive association is not significant statically) (Table 4).

Table 4: The association between TNF-α expression and clinicopathologic features (n = 60).

Infiltrative Ductal Carcinoma (IDC)		Number of Negative TNF- α Expression - %	Number of Positive TNF-alpha Expression - %			
			Score 1 Weak < 25%	Score 2 Moderate 25–50%	Score 3 Strong, 51-75%	Score 4 Strong, >75%
Grade	Grade I	2 (28.59)	1 (33.33)	1 (11.11)	4 (18.18)	3 (15.78)
	Grade II	4 (57.14)	1 (33.33)	6 (66.66)	14 (63.63)	11 (57.89)
	Grade III	1 (14.28)	1 (33.33)	2 (22.22)	4 (18.18)	5 (26.31)
	P value	0.972				
Pathological stage	T1N0	2 (28.59)	1 (33.33)	1 (11.11)	4 (18.18)	**0**
	T2N0	1 (14.28)	1 (33.33)	1 (11.11)	5 (22.72)	2 (10.52)
	T2N1	**0**	**0**	1 (11.11)	5 (22.72)	5 (26.31)
	T2N2	**0**	**0**	6 (66.66)	1 (4.54)	4 (21.05)
	T3N0	**0**	**0**	**0**	1 (4.54)	**0**
	T3N1	1 (14.28)	1 (33.33)	**0**	1 (4.54)	2 (10.52)
	T3N2	3 (42.85)	**0**	**0**	2 (9.08)	2 (10.52)
	T4N0	**0**	**0**	**0**	2 (9.08)	1 (05.26)
	T4N1	**0**	**0**	**0**	1 (4.54)	3 (15.78)
	P value	0.081				
TNM stage	I	2 (28.59)	1 (33.33)	1 (11.11)	4 (18.18)	**0**
	IIA	1 (14.28)	1 (33.33)	1 (11.11)	5 (22.72)	2 (10.52)
	IIB	**0**	**0**	1 (11.11)	6 (27.26)	5 (26.31)
	IIIA	4 (57.14)	1 (33.33)	6 (66.66)	4 (18.18)	8 (42.10)
	IIIB	**0**	**0**	**0**	3 (13.63)	4 (21.05)
	P value	0.286				
Tumor size (cm)	=<2 cm	2 (28.59)	1 (33.33)	1 (11.11)	4 (18.18)	**0**
	>2cm – 5cm	5 (71.41)	2 (66.66)	8 (88.88)	13 (59.09)	8 (42.10)
	>5cm	**0**	**0**	**0**	5 (22.72)	11 (57.89)
	P value	0.210				
Lymph Node status	Positive	4 (57.14)	1 (33.33)	7 (77.77)	10 (45.45)	16 (84.20)
	Negative	3 (42.85)	2 (66.66)	2 (22.22)	12 (54.54)	3 (15.78)
	P value	0.070				

DISCUSSION

The role of TNF-α in tumor activation and cancer progression is controversial. Tumor necrosis factor alpha (TNF-α), isolated 30 years ago, is an extraordinarily pleiotropic cytokine, that plays important roles in diverse cellular events, cell survival, proliferation, differentiation, and death, with a central role in immune homeostasis, inflammation, and host defense. TNF can induce apoptosis, necrosis, angiogenesis, immune cell activation, and cell migration. Although named for its antitumor properties, TNF has been implicated in tumor development and tumor progression. In regard to cancer, TNF could be an endogenous tumor promoter, through stimulation of cancer cells' growth, proliferation, invasion and metastasis, and tumor angiogenesis. On the other hand, TNF displays pro- and antitumoral effects.[18,19] The interaction of TNF-α with TNF receptor 1 and receptor 2 (TNFR-1, TNFR-2) activates several signal transduction pathways, leading to the diverse functions of TNF-α. The signaling molecules of TNFR-1 have been elucidated quite well, but regulation of the signaling still unclear.[20] TNF exerts its biological functions through activating distinct signaling pathways such as nuclear factor κB (NF-κB) and c-Jun N-terminal kinase (JNK). NF-κB is a major cell survival signal that is anti-apoptotic while sustained JNK activation contributes to cell death. This relation between the NF-κB and JNK is involved in determining cellular outcomes in response to TNF.[18] Many studies documented a pro-tumorigenic role of TNF-α in vivo, in part by suppressing necrosis and by activation a proangiogenic myeloid phenotype.[21,22] Besides extravasation of erythrocytes and lymphocytes, leading to hemorrhagic necrosis, TNF-α targets the tumor-associated vasculature (TAV) by inducing hyperpermeability and destruction of the vascular lining. This results in an immediate effect of selective accumulation of cytostatic drugs inside the tumor and a late effect of destruction of the tumor vasculature.[20] Balkwill (2006), discussed the involvement of TNF-α in the inflammatory network that contributes to all stages of the malignant process, and considered the possibility that TNF-α be a target for cancer therapy.[15] With better understanding of the molecular mechanisms of TNF-induced cellular signaling, it is getting clear that TNF plays a major role in the development and progression of different types of cancer.[18] However, TNF role in cancer biology is still under discussion. In spite of the many evidence supporting its antitumor activity, the molecular events of TNF-mediated tumor regression observed in vivo is still incompletely clarified.[23]

The present routine assessment of breast cancer depends on use of few biological markers and clinicopathological variables (histological grade, stages, and lymph node involvement) [24, 25]. With this work we intend to

summarize the intensity and degree of expression of TNF-α in primary breast carcinoma and its correlation with different clinicopathologic features, (with particular regard to pathological stage, tumor grade and lymph node status). And review the clinical and experimental evidence describing the conflicting relationship of this cytokine and cancer biology.

The present results revealed a statistically significant expression of TNF-α in malignant tumors in comparison with the benign tumors (p=0.05). Positive expression of TNF-α is identified in most cases of breast carcinoma (88.33%), (Sensitivity=88.33%; false negative=11.66%; positive predictive value=88.33%). While 7 benign cases were positive for TNF-α (46.66%), (Specificity=53.33%; False Positive%=46.66%; Negative Predictive value=53.33%) and the accuracy rate is 81.33%. The false positive and false negative may be a result of procedure troubleshot such as laboratory conditions that may affect the intensity and positivity of the stain. In this study, the expression of TNF-α among malignant tumors is slightly higher in that with higher grade, higher stage and positive lymph node metastases compared to that of lower grade, lower stage, and negative lymph node involvement. This association is not significant statically (p value more than 0.05).

These results may point to the role of this marker in growth of breast carcinoma, as well tumor invasion and metastasis, perhaps other tumor markers are playing a significant role in invasion and metastasis of breast cancer, or may induced other factors inside cells that lead to activation and promotion of tumor angiogenesis and then lead to spreading malignant cell to other part of organs and body. Other workers showed a significant correlation between TNF-α and clinicopathological expression. The account of tumor cells which expressing TNF-α in breast carcinoma was showed to be associated with tumor grade and lymph node involvement, as well the TNF-α expression was suggested to play a pivotal role in the spreading of breast tumors[17]. Additionally, studies of this markers suggested that the strong expression of TNF-α in breast tumors actually promote tumor growth. Berberoglu and co workers (2004), reported high serum TNF-*α* in patients with advanced stages of breast carcinoma and this serum level correlated well with number and size of metastatic sites [26]. Study by Blackwill, (2006), revealed that TNF-α play important role in promotion and progression of cancer by regulating pathways that lead to cell proliferation, survival and angiogenesis [21]. Other studies describe a temporal correlation between growing of skin tumors and lymphoma and the use of TNF-α inhibitors [27, 28]. Studies on mouse cells (in vivo) and on patients with cancer offering key role of TNF-α in tumor growth [29]. Besides these molecular insights, laboratory experiments in the past decade have shed light upon TNF-α action during tumor treatment. The use of TNF in cancer is in the regional treatment of locally advanced soft tissue sarcomas and metastatic melanomas and other irresistible tumors of any histology to avoid amputation of the limb. It has been demonstrated in the isolated limb perfusion setting that TNF-α acts synergistically with cytotic drugs [20]. Modulation of the activity of the TNF-TNF receptor system offers manifold possibilities for cancer therapy. TNF and in combination with Melphalan is a treatment option for soft tissue sarcoma and data suggest that TNF neutralization could also be exploited to fight cancer complications [19].

CONCLUSION

Our results showed that TNF-α protein is strongly positive expressed in breast cancer and is positively correlated with the malignant breast tumors. These findings give us an indication of TNF-α might play a role in the proliferation of breast cancer cells, impartial of lymph node involvement, grade and stages of the tumor.

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CONTRIBUTIONS

Ban J M designed the study, provided the TNF antibody kit as well as all necessary solutions & materials, in addition to performing of staining steps. Reyadh S M provided the tissue sections and statistics, while Faeza A Z scanned and scored the expression of TNF in tissue sections plus she wrote and revised the final version.

Conflict of Interest: None.

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