The Egyptian Journal of Hospital Medicine (January 2025) Vol. 98, Page 283- 288

Non-Metastatic Triple Negative Breast Cancer with Neuroendocrine Differentiation: Survival Parameters

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ABSTRACT

Background: A testing for neuroendocrine markers during breast cancer diagnosis is rarely required. Also, breast cancer with neuroendocrine differentiation is highly under presented in clinical trials.

Objective: This research aimed to study patients with triple negative breast cancer (TNBC) with neuroendocrine differentiation to outline their characteristics and outcomes.

Patients and methods: We examined effect of synaptophysin expression and other clinicopathological features of 35 non-metastatic triple negative breast cancer females for disease free survival (DFS) and overall survival (OS).

Results: There was a statistically significant association between synaptophysin-enriched expression and tumor infiltrating lymphocytes (TILs) > 50% in TNBC patients (P= 0.047). Stage III disease, tumor size ≥ T3, high Ki67 and presence of ductal carcinoma in situ (DCIS) were significantly associated with decreased DFS. Tumor size ≥ T3 and high Ki67 were bad prognostic factors for overall survival. Of note, Ki67 was the sole independent prognostic factor predicting DFS and OS.

Conclusion: There is a clear association between synaptophysin expression and increased TILs percentage in TNBC raising the possible benefit of immunotherapy in this category of breast cancer, however, further study with larger sample size is needed for outlining the survival effects of synaptophysin expression.

Keywords: Breast cancer, TNBC, Neuroendocrine differentiation, Synaptophysin.

INTRODUCTION

Breast cancer has the highest incidence of cancer diagnosis among women constituting for about 2.3 million cases yearly [(1, 2)]. Breast cancer is a diverse disease regarding clinical, pathological, and molecular patterns [(3)]. Triple negative breast cancer (TNBC) is the least common biological subtype of breast cancer constituting 10-15 % of all breast cancer patients. This type lacks ER, PR and HER2 expression. This type is characterized by an aggressive biological behavior represented in highest recurrence and distant metastasis rates and poorest survival outcome among all types of breast carcinoma [(4)].

Synaptophysin is one of the neuroendocrinal markers. It is routinely required in many cancers especially in small cell lung cancer [(5)].

Unfortunately, it is rarely put in the panel of diagnosis of breast cancer [(6)]. Consequently, there is a paucity of studies conducted to investigate the effect of Synaptophysin expression in patients with breast cancer specially TNBC [(6)].

Therefore, we conducted this study to investigate the association of synaptophysin expression with other clinicopathological features and the survival outcome in TNBC patients.

PATIENTS AND METHODS

Study design and patients: A retrospective study of 35 non-metastatic TNBC females aged 18 years or more received treatment during the period between January 2015 and December 2022 in Medical Oncology Department, South Egypt Cancer Institute, Assiut University. Excluding criteria include patients with incomplete data or double malignancy.

Breast cancer staging and management: Study patients were staged with appropriate baseline assessment, including history taking, clinical examination, radiological investigations accordingly (CT/MRI ± bone scan). Complete pathological data were collected. All received neoadjuvant and/or adjuvant chemotherapy were reported. Patients are stratified according to their age, menopausal status, BMI, stage of disease, grade of differentiation, tumour size, nodal involvement, lymphovascular invasion (LVI), perineural invasion (PNI) and ductal carcinoma in situ component (DCIS). Tumor infiltrating lymphocytes (TILs) had two categories: Non-brisk immune response (TILs < 50%), brisk immune response (TILs > 50%). [(7)]

Survival outcomes as follows: DFS defined as the period from date of surgery to date of recurrence or death, while OS defined as the period from date of surgery to date of death.

Synaptophysin immunohistochemistry: The 35 formalin-fixed paraffin-embedded (FFPE) tissue blocks of TNBC and one control block of resected pyloric neuroendocrine tumor diffusely positive synaptophysin expression were obtained from the archives of the Oncologic Pathology Department, SECI. Each block was cut into 5 µm thick sections and mounted on positively charged slides.

1. **Rehydration:** Rehydration done through putting slides in xylene for 10 minutes then alcohol 100% for 5 minutes then alcohol 95% for 5 minutes then alcohol 70% for 5 minutes then dill water.

Received: 08/08/2024
Accepted: 08/10/2024

2. **Antigen retrieval:** Heat slides in Ag retrieval solution (tris EDTA PH9) at 95° -97° C for 20 minutes, then let slides to cool.

3. **Staining:**

1) Put primary Antibody: Primary Synaptophysin ZM208 Mouse monoclonal antibody solution (65 N 1st Ave, Ste 202C, Arcadia, CA91006, USA) was applied into tumor tissue sections. The tissue slices were then incubated for 16 hour at room temperature.
2) Secondary AB:
   a) Primary AB enhancer: applied for 15 minutes then wash by PBS.
   b) Zeta Universal HRP Polymer Detection Kit with DAB Chromogen (Anti-Mouse-HRP + Anti-Rabbit-HRP) was applied for 30 minutes then washed by PBS.
3) Zeta Universal HRP polymer Detection Kit with DAB chromogen (DAB substrate, High contrast). 1 ml DAB substrate ~ 1 drop DAB chromogen 5 minutes then wash by PBS.
4) Hematoxyline Mayer's stain for 10 minutes then wash by PBS then wash by dill water.
5) Dehydration: Dehydration by exposure to alcohol 70% then alcohol 95% then alcohol 100%.
6) Mountain covers.
7) Examination under microscope.

Evaluation of synaptophysin expression:

A modified Allred score was used, which is sum of Allred cell percentage and intensity of Synaptophysin expression scores, summation product 0 – 8, Synaptophysin enriched expression considered if score is 6 – 8, while considered non-enriched expression if score is 0 – 5 [Table 1] [8].

Table (1): Modified Allred score for Synaptophysin expression

Proportion score A	Positive cells, %	Intensity	Intensity score B
0	0	None	0
1	<1	Weak	1
2	1 to 10	Intermediate	2
3	11 to 33	Strong	3
4	34 to 66	Final score range (A + B): 0-8	
5	≥67		

Ethical approval: Ethical approval of Institutional Ethical Committee numbered as SECI- IRB IORG000563-601. Following receipt of all information, signed consent was provided by each participant. The study adhered to the Helsinki Declaration throughout its execution.

Statistical methods:

All statistical calculations were done using SPSS version 22. Kaplan-Meier's method with log rank test used for disease free and overall survival analysis. Hazard ratio (HR) with 95% Confidence Interval (CI) and COX regression analysis was calculated to determine significant factors associated with recurrence and mortality. P-value is always 2 tailed set significant at 0.05 level. For comparing categorical data, Chi square ($\chi 2$) or Fisher Exact test (when appropriate) was performed. P-value is always 2-tailed set and considered significant if less than 0.05 level.

RESULTS

Regarding association of Synaptophysin with other clinicopathological criteria, only TILs showed statistically significant association, as 40.0% of patients with TILs more than 50% had Synaptophysin enriched tumor versus only 3.3% in those with TILs less than 50% as shown in table (2).

Table (2): correlation between patient clinicopathological criteria and Synaptophysin expression status

Patients characteristics		Synaptophysin expression				
		Non enriched (n=32)		Enriched (n=3)		
		N	(%)	N	(%)	P value
Age	< 50 years	12	100.0%	0	0.0%	0.536
	≥ 50 years	20	87.0%	3	13.0%	
Menopausal status	Premenopausal	12	100.0%	0	0.0%	0.536
	Postmenopausal	20	87.0%	3	13.0%	
BMI category	< 30	18	94.7%	1	5.3%	0.582
	≥ 30	14	87.5%	2	12.5%	
Stage	Stage I, II	12	85.7%	2	14.3%	0.551
	Stage III	20	95.2%	1	4.8%	
Grade	Grade II	31	91.2%	3	8.8%	1.000
	Grade III	1	100.0%	0	0.0%	
Tumor size	< T3	19	90.5%	2	9.5%	1.000
	≥ T3	13	92.9%	1	7.1%	
Lymph nodes	Node negative	8	88.9%	1	11.1%	1.000
	Node positive	24	92.3%	2	7.7%	
Ki67	Low	11	84.6%	2	15.4%	0.541
	High	21	95.5%	1	4.5%	
Ductal carcinoma in situ	No	19	90.5%	2	9.5%	1.000
	Yes	13	92.9%	1	7.1%	
Lymphovascular invasion	No	7	100.0%	0	0.0%	1.000
	Yes	25	89.3%	3	10.7%	
Perineural invasion	No	25	92.6%	2	7.4%	0.553
	Yes	7	87.5%	1	12.5%	
TILs	Less than 50 %	29	96.7%	9	3.3%	**0.047**
	More than 50 %	3	60.0%	2	40.0%	
No. of metastatic sites	≤ 2	28	90.3%	3	9.7%	1.000
	> 2	4	100.0%	0	0.0%	
Chemotherapy	Anthracycline based	31	91.2%	3	8.8%	1.000
	Non anthracycline	1	100.0%	0	0.0%	
	Single agent	6	85.7%	1	14.3%	0.511
	Combination	25	92.6%	2	7.4%	

Data are presented as number (percentage). Significance defined by P-value ≤ 0.05. Abbreviations: LVI, Lymphovascular Invasion; PNI, Perineural Invasion; DCIS, Ductal Carcinoma In Situ; TIL, Tumor Infiltrating Lymphocytes.

Regarding DFS and OS, there was no statistically significant difference between patients with Synaptophysin non-enriched and those with enriched expression as shown in figures (1).

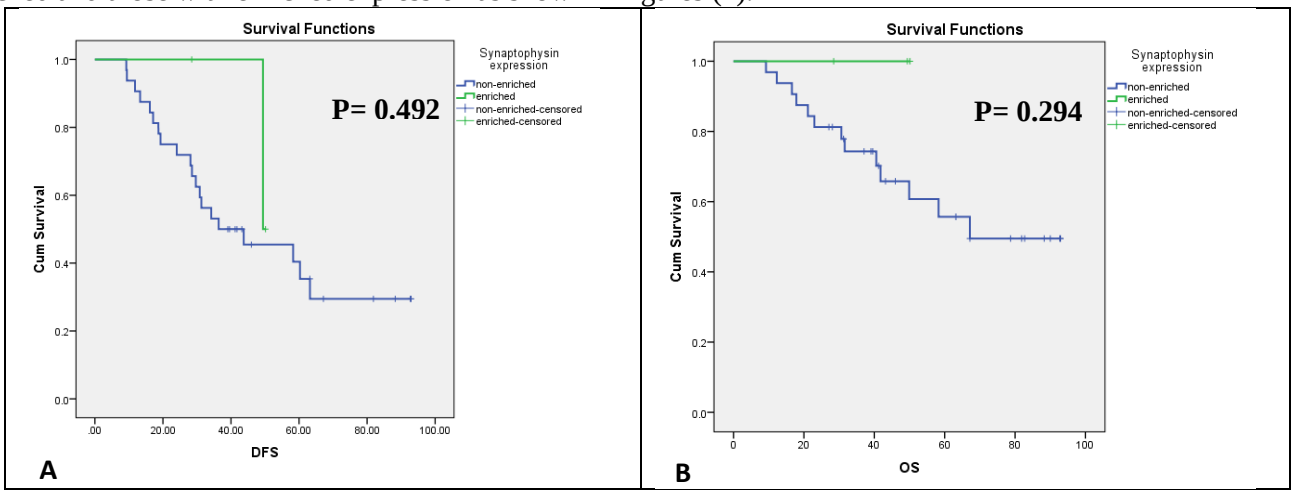

Figure (1): Effect of Synaptophysin expression on DFS (A) and OS (B) of triple negative BC.

Univariate and multivariate analysis for DFS of TNBC patients: In univariate analysis, stage III disease, tumor size ≥ T3, high Ki67, presence of DCIS was associated with decreased disease free survival. As, stage III disease increased risk of recurrence or death by four folds (HR= 4.043, 95% CI = 1.451-11.260, P= 0.008). While, tumor size ≥ T3 increased risk of recurrence or death by 2.5 folds (HR= 2.567, 95% CI = 1.071-6.151, P= 0.034). Regarding Ki67, high Ki67 increased risk of recurrence or death by 7.5 folds (HR= 7.552, 95% CI = 2.109-27.037, P= 0.002). Notably, the presence of DCIS had been associated with increased recurrence by 2.8 folds (HR= 2.873, 95% CI = 1.068-7.728, P= 0.037). Moreover, in multivariate analysis, high Ki67 was the only independent factor predicting increased recurrence or death rate (HR= 6.423, 95% CI = 1.734-23.794, P= 0.005). (Table 3).

Table (3): COX regression analysis for prediction of recurrence among the studied TNBC cases

Variables	Univariate analysis			Multivariate analysis		
	HR	**95% CI**	**P value**	**HR**	**95% CI**	**P value**
Age						
• **< 50 yrs**	1.665	0.684- 4.052	0.262			
• **≥ 50 yrs**	ref					
Tumor stage						
• **Stage I, II**	ref			ref		
• **Stage III**	4.043	1.451-11.260	**0.008**	1.885	0.519-6.842	0.335
Tumor size (cm)						
• **< T3**	ref			ref		
• **≥ T3**	2.567	1.071-6.151	**0.034**	1.516	0.547-4.204	0.424
Ki67						
• **Low**	ref			ref		
• **High**	7.552	2.109-27.037	**0.002**	6.423	1.734-23.794	**0.005**
DCIS						
• **No**	ref					
• **Yes**	2.873	1.068-7.728	**0.037**	2.303	0.795-6.674	0.124
TILs						
• **<50%**	4.753	0.636-35.540	0.129			
• **>50%**	ref					

Univariate and multivariate analysis for OS of TNBC patients: In Univariate analysis, tumor size ≥T3 and high Ki67 was associated with increased risk of death. As, tumor size ≥T3 increased risk of death by 3.7 folds (HR= 3.765, 95% CI = 1.215-11.671, P= 0.022). Regarding Ki67, high level increased risk of death by 18 folds (HR= 18.641, 95% CI = 2.238-155.254, P= 0.007). With multivariate analysis, the high Ki67 was the only independent factor predicting increased risk of death (HR= 18.030, 95% CI = 2.006-162.031, P= 0.010) (Table 4).

Table (4): COX regression analysis for prediction of death among the studied TNBC cases

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	P value	HR	95% CI	P value
Age						
• **< 50 yrs**	1.179	0.356- 3.909	0.788			
• **≥ 50 yrs**	ref					
Stage						
• **I, II**	ref					
• **III**	59.016	0.666-5228.844	0.075			
Tumor size (cm)						
• **< T3**	ref			ref		
• **≥ T3**	3.765	1.215-11.671	**0.022**	2.988	0.928-9.622	0.067
Nodal involvement						
• **Node negative**	ref					
• **Node positive**	34.503	0.211-5640.060	0.173			
Ki67						
• **Low**	ref			ref		
• **High**	18.641	2.238-155.254	**0.007**	18.030	2.006-162.031	**0.010**
TILs						
• **<50 %**	26.538	0.043-16430.155	0.317			
• **>50%**	ref					

DISCUSSION

Triple negative breast cancer (TNBC) is the least common biological subtype of breast cancer constituting 10-15 % of all breast cancer patients. This type lacks ER, PR and HER2 expression so not benefiting from their targeting treatment agents with aggressive biological behavior represented in higher recurrence and distant metastasis rates and poor survival outcome [(4)]. The breast with neuroendocrine differentiation is highly under recognized due to absent routine testing for neuroendocrine markers in breast cancer diagnosis. Consequently, TNBC with neuroendocrine differentiation still an ambiguous issue [(6)].

In the current study, the only revealed association of synaptophysin expression is with tumor infiltrating lymphocytes, as 40.0% of patients with TNBC that had TILs more than 50% were Synaptophysin enriched while 3.3% only of those with TILs less than 50%. Stage III disease, Tumor size ≥T3, high Ki67 and presence of DCIS was associated with increased incidence of recurrence. In addition, tumor size ≥ T3 and high Ki67 was associated with increased incidence of death in TNBC patients. High Ki67 is the only independent prognostic factor for recurrence and death. Regarding DFS and OS, there was no statistically significant difference between patients with Synaptophysin non-enriched and those with enriched expression.

As regards the correlation of synaptophysin expression status with TILs, it is not described before in previous studies so need further investigation in a larger cohort study to support this finding. The higher TNM stage and tumor size associated with lower DFS also described by **Cia *et al.*** [(9)] study of clinicopathological data of 1584 TNBC patients that described increased rate of distant and local recurrence with higher T staging, N staging, TNM staging, and low expression of stromal tumor-infiltrating lymphocytes (sTILs) [(9)].

larger tumor size associated with shorter disease free survival and overall survival in the current study is promoted by the finding of **Lai *et al.*** [(10)] study who reported that tumors 1 cm and smaller, lymph node invasion was the single most important prognostic factor for DFS and CSS.

In this study, age below or above 50 yrs was not associated with statistically significant difference in incidence of recurrence, which is supported by the finding of **Radosa *et al.*** [(11)] study that reported young age at diagnosis is not an independent risk factor for local or distant recurrence in patients with TNBC. But, this result is in contrast to the finding of **Vihervuori *et al.*** [(12)] study that reported age at diagnosis as the critical factor for mortality in one hundred forty seven TNBC patients ($p = 0.002$).

High Ki67 in our study, is the only independent factor predicting higher recurrence and death rate that is coinciding with the finding of **Li *et al.*** [(13)] who classified 1146 stage I, II TNBC patients according to Ki67 level and reported that Ki-67 level of > 45% was associated with poorer disease-free survival ($p = 0.039$) and overall survival ($p = 0.029$).

Limitations of our study are its retrospective nature and small sample size; however, it is statistically acceptable for reliability.

CONCLUSION

There was a clear association between synaptophysin expression and increased TILs percentage in TNBC raising the possible benefit of immunotherapy in this category of breast cancer. However, further study with larger sample size is needed for outlining the survival effects of synaptophysin expression.

No funding.

No conflict of interest.

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