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THE EXPRESSION LEVEL OF T-BOX TRANSCRIPTION FACTOR TBX2 IN BREAST CANCER AND ITS CLINICAL SIGNIFICANCE

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Abstract – *Objective:* *Recent studies showed that overexpression of T-box transcription factor TBX2 in ovarian and prostate cancer tissues is associated with conferred resistance to chemotherapeutic drugs. However, whether TBX2 is overexpressed in breast cancer tissues remains to be elucidated. The present study aimed to determine the level of TBX2 in breast cancer tissues in comparison to normal tissues and to study the association between the level of TBX2 and breast cancer metastasis.*

Patients and Methods: *Breast cancer samples and their adjacent non-tumor tissues were collected from 51 breast cancer patients. Sections from each sample were immune-stained by anti-TBX2 and suitable secondary and tertiary antibodies. TBX2 levels were evaluated in cancerous and non-tumor samples. TBX2 level was also evaluated by Western blotting. The correlation between TBX2 levels and the clinicopathological parameters was statistically tested.*

Results: *Data showed that TBX2 is significantly overexpressed in breast cancer tissues compared with non-tumor adjacent tissues. Furthermore, it showed that TBX2 expression is associated with cancer metastasis and lymph node size. Statistical analysis showed that TBX2 overexpression is a promising biomarker for breast cancer cells with an area under the curve [AUC] of 0.891 [p<0.001] at a cut-off value of 10 of a total score of 16.*

Conclusions: *TBX2 is overexpressed within breast cancer tissues, and increased levels of this transcription factor can be found in breast cancer cells for patients with metastatic disease suggesting that TBX2 might serve as a useful diagnostic marker in this malignancy.*

KEYWORDS: *TBX2 transcription factor, Breast cancer diagnosis, Metastasis.*

INTRODUCTION

TBX2 is a T-box transcription factor that is overexpressed in several cancers and its expression is associated with the clinical stage and pathological grade of the tumor[1-19]. For example, 52% of cervical squamous cell carcinoma tissue sections tested showed high levels of TBX2, and these levels were significantly higher in sections of Human Papillomavirus [HPV]-16 positive tumors[20]. Consistent

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with these findings, metastatic pancreatic, ovarian, and prostate cancer tissues also showed elevated TBX2 levels compared with non-tumor adjacent tissues[17,18]. Furthermore, TBX2 expression was also associated with clinical and pathological parameters. Regarding the TBX2 level in breast cancer, several breast cancer cell lines showed amplified levels of TBX2[13,14,21,22]. Interestingly, *TBX2* was reported to be amplified at a small number of breast carcinomas and it was preferentially amplified in breast cancer gene 1 (*BRCA1)* and breast cancer gene 2 (*BRCA2)* associated breast cancers[14]. Together, these observations show that TBX2 plays an important role in cancer cell proliferation metastases suggesting it as a useful marker for breast cancer. In a continuation of another project aimed to explore the significance of TBX3 as a diagnostic marker for breast cancer [23], here we determined the TBX2 level in a population of breast cancer patient's tissues in comparison to non-tumor tissues. The diagnostic value of TBX2 as a biomarker for breast cancer and the association between TBX2 with the clinical and pathological parameters were also tested.

PATIENTS AND METHODS

Breast cancer samples

A total of 51 breast cancer patients who had undergone mastectomy at the European Gaza Hospital were enrolled in this study. The research protocol was Ethically approved by the Helsinki Committee of the Palestinian Health Research Council. ***Table S1*** describes the pathological and clinical characteristics of the population. Paraffin-embedded formalin-fixed blocks for tumor and non-tumor tissues for each patient were included in the study. Informed consent was given by each patient involved in the study.

Immunohistochemistry

Three 4 µm sections of each submitted paraffin block were deparaffinized and rehydrated using a xylene and ethyl alcohol. Trilogy (Cell Marque™; Merck KGaA, Darmstadt, Germany) and hydrogen peroxide were used for retrieval, blocking, hydration, and washing. One section for each block was first stained with hematoxylin-eosin (HE) to verify the pathological diagnosis and to validate the protocol quality. The other two sections were incubated with anti-TBX2 primary antibody (Ab33298, Abcam, Cambridge, UK) 1:150 dilution, then the primary antibody was detected by HRP HiDef 2-Step Polymer Detection™ Kit (954D, Cell Marque, Merck KGaA, Darmstadt, Germany). The DAB chromogen Kit (957D, Cell Marque, Merck KGaA, Darmstadt, Germany) was used for all reactions. Negative control sections were prepared similarly, excluding the TBX2 antibody. All sections were stained with hematoxylin and examined using an iScan Coreo Digital Microscope (Version 3.3, Ventana, AZ, USA)[24].

The scoring method for TBX2 expression

Expression of TBX2 in both tumor and non-tumor tissues was scored as previously described[23,25]. Briefly, nuclear and cytoplasmic staining were evaluated in 20 normal cells of the non-tumor sections or 20 tumor cells of the tumor sections. Both the staining intensity-based system and the proportion of stained cells-based system were used. The nuclear staining was classified into a proportion score (0-5) and intensity score (0-3). The cytoplasmic staining was also classified into a proportion score (0-5) and intensity score (0-3). A total score (0 to 8) was given to describe the nuclear and cytoplasmic staining separately. By adding both cytoplasmic and nuclear scores together, the total TBX2 score was 0 to 16.

Immunoblotting

Western blot was used to detect the expression level of TBX2 in breast cancer and non-tumor tissue samples. Tissues were homogenized in a lysis buffer supplemented with a cocktail of protease inhibitors. Then, 30 µg protein were separated with electrophoresis on an 8% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membranes were incubated overnight at TBX2 antibody (1:1000) or β-actin antibody (1:2000). Then membranes were washed and incubated in the appropriate secondary antibodies. The antibody- reactive proteins were visualized using the chemiluminescence reaction (ECL) detection system (Thermo Scientific, Hudson, NH, USA)[26].

Statistical Analysis

Standard statistical techniques were used to analyze the TBX2 expression in tumor and non-tumor tissues of breast cancer patients. Statistical Package for Social Sciences (SPSS) software package version 22.0 (SPSS Corp., IBM, Armonk, NY, USA) was used for all statistical analyses. Personal information variables were presented as percentages and frequencies. The *t*-test was used to compare between

TBX2 level in both tumor and non-tumor tissues. Spearman's rank correlations test and Mann-Whitney U-test (MW) were used to investigate the relationship between TBX2 expression and the clinical and pathological features of breast cancer patients. To analyze the relationship between TBX2 level and the clinical features of different breast cancer groups based on stages (I, II, III, and IV) and histological grades (1, 2, and 3), Kruskal-Wallis H-test (KW) was used and p-values < 0.05 were considered to be statistically significant. The TBX2 Diagnostic accuracy was determined by the Receiver Operating Characteristic Curve (ROC). The cut-off value and the Area Under a ROC Curve (AUC) were used to test the significance of nuclear, cytoplasmic, and total TBX2 levels as a tumor marker. The values of the maximum sum of sensitivity and specificity were considered as the optimal cut-off values.

RESULTS

TBX2 is overexpressed in breast cancer tissues and correlated with cancer metastasis

Recent studies indicated that breast cancer tissues have high levels of TBX2[12,14,27,28]. To explore this in a cohort of breast cancer patients, an immunohistochemistry assay was performed on 51 breast cancer tissues and 40 blocks of adjacent non-tumor tissues. Tumor and non-tumor sections were stained and scored as described in the patients and methods section[25,29,30]. The results displayed that the TBX2 protein level in cancer cells is overexpressed compared with non-tumor cells (p<0.001, t=8.454) (Figure 1a-1d).

Indeed, TBX2 was overexpressed in 25 breast cancer tissues (62.5%) and only 4 (10%) of non-tumor tissues showed similar levels of TBX2. While only one breast cancer sample (2.5%) showed a weak TBX2 level, 11 samples (27.5%) of non-tumor tissues showed a weak TBX2 level. Moreover, no tissues showed a completely negative TBX2 level. The expression levels of TBX2 in non-tumor and tumor tissues are summarized in Table 1. The mean of TBX2 level in cancer tissues is 12.4±2.7 and in non-tumor tissues is 6.5±4.2 (p<0.001). These

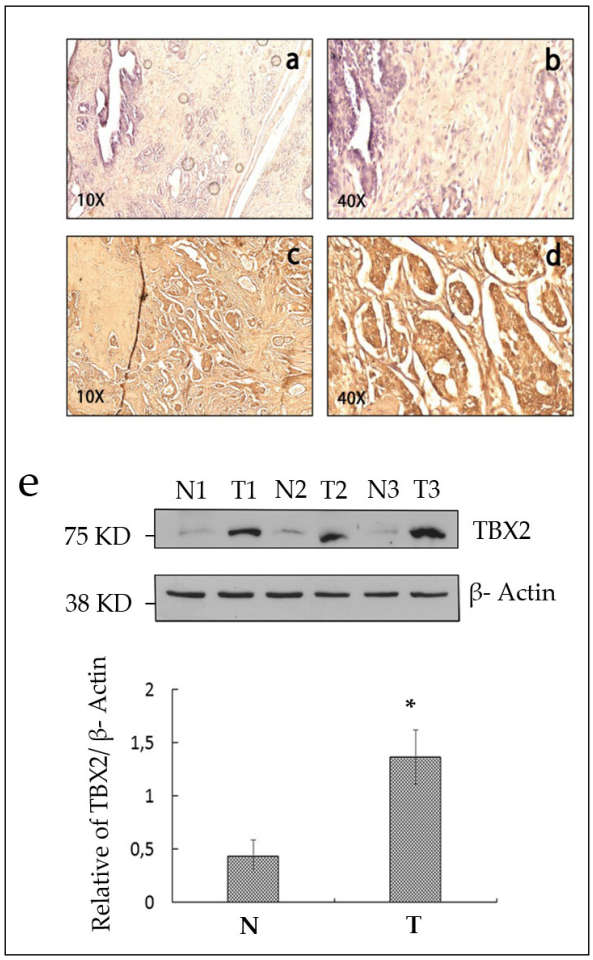

Fig. 1. TBX2 level in breast cancer tissues and adjacent non-tumour tissues. Non-tumour human breast epithelial tissues [a] at 10X and [b] 40X. Breast cancer tissues [c] at 10X and [d] at 40X. Representative photos of Western blot for the expressions of TBX2 and β-actin in tumor and non-tumor tissues [e]. The graph shows the densitometry analysis of expressed TBX2 and β-actin in tumor and non-tumor tissues as calculated by Image J program. *p<0.05.

results were also confirmed by Western blotting where TBX2 level was overexpressed in tumor tissues in comparison to non-tumor tissues (1.3±0.25 in tumor tissues *vs.* 0.4±0.14 in non-tumor tissues) (Figure 1e).

Importantly, our results revealed that TBX2 is mainly cytoplasmic in both non-tumor and breast cancer tissues. According to our scoring system (cytoplasmic TBX2 level (0-8) and (nuclear TBX2

TABLE 1. TBX2 expression levels in tumour and non-tumour tissues.

Tissues	*Sample number*	*Degrees of TBX2 expression*				
		Negative %	*Weak %*	*Moderate %*	*Strong %*	*p-value*
Non-tumour	40	0 [0%]	11 [27.5%]	25 [62.5%]	4 [10%]	<0.001
Tumour	40	0 [0%]	1 [2.5%]	14 [35%]	25 [62.5%]	<0.001

TABLE 2. The table shows the association between TBX2 level and Lymph node size and metastasis.

Characteristic	Sum nuclear and cytoplasm TBX2		χ^2	p-value
	≤10 n (%)	>10 n (%)		
Tumor size (cm) (n=48)				
≤ 2	1 (7.1)	3 (8.8)	1.027	0.598
2-5	8 (57.1)	14 (41.2)		
> 5	5 (35.7)	17 (50)		
LN size (cm) (n=43)				
≤1	7 (58.3)	7 (22.6)	5.036	0.025
>1	5 (41.7)	24 (77.4)		
LN met (%) (n=49)				
0	5 (35.7)	14 (40)	8.693	0.034
1-3	4 (28.6)	8 (22.9)		
4-9	5 (35.7)	3 (8.6)		
>10	0 (0)	10 (28.6)		

level (0-8)), the mean of cytoplasmic TBX2 level in non-tumor cells is 4±2.5 compared with 2.5±2 in the nucleus and this difference was significant with $p<0.001$, (***Table S2***). Similarly, the mean of cytoplasmic TBX2 level in tumor cells is 6.8±1.5 compared with 5.6±1.9 in the nucleus and this was also significant $p<0.001$ (***Table S3***). Our results showed that there are significant positive correlations between TBX2 levels and lymph node size and metastasis $p<0.05$ (Table 2). We observed that tumors expressing a high level of TBX2 showed high lymph node size and increased tendency toward metastasis. Importantly, 28.6% of patients with high TBX2 tumors have more than 10 affected lymph nodes. Regarding lymph node size, 77.4% of patients with high TBX2 tumors have large lymph node size (more than 1 cm) and these results are also statistically significant $p<0.05$. However, we didn't observe any significant correlation between TBX2 levels and other clinical and pathological parameters such as estrogen receptors (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), breast cancer type, stage, and grade.

TBX2 is a promising biomarker for breast cancer tissues

To explore the significance of TBX2 as a biomarker of breast cancer tissues we analyzed the obtained data using the ROC curve and AUC value. Youden's index was used to determine the cut-off value for TBX2 level in tumor tissues compared to normal tissues. The results showed that TBX2 protein is a potential biomarker to distinguish between breast cancer and non-tumor tissues ($p<0.001$). The-cut off value for the TBX2 level was 10 from a total score of 16, the AUC was 0.891 ($p<0.001$), sensitivity and specificity were 72.5% and 88.2%, respectively. Positive predictive value (PPV) was 86%, negative predictive value (NPV) was 76.3%, and accuracy was 80.4% to diagnose breast cancer. The cut-off value for cytoplasmic TBX2 was 6 from a total score of 8, the AUC was 0.833 ($p<0.001$), sensitivity was 70.6%, specificity was 80.4%, PPV was 78.3, NPV was 73.2, and accuracy was 75.5% to diagnose breast cancer. Regarding nuclear TBX2, the cut-off value was 3 from a total score of 8, the AUC was 0.862 ($p<0.001$), sensitivity was 78.4%, specificity was 80.4%, PPV was 80, NPV was 78.8, and accuracy was 79.4% to diagnose breast cancer (***Table S4***). In conclusion, these findings showed that total level of TBX2 might represent a potential biomarker for breast cancer tissues with an accuracy of 80.4% and specificity of 88.2%, and AUC of 0.891 ($p<0.001$) (Figure 2).

DISCUSSION

TBX2 is a member of the T-box transcription factors family that plays critical roles in the development and oncogenic process[31]. Compelling evidence indicated a functional linkage between TBX2 and cancer cell proliferation and migration[17,32,33]. Several studies showed that TBX2 is overexpressed in a wide range of carcinomas like ovarian, cervical, prostate, and melanoma[32]. Recent findings revealed that silencing of TBX2 in more than one kind of aggressive human cancer cell line led to inhibit EMT and tumor cell migration[9,32,34]. Based on the above data we assumed that TBX2 might be expressed similarly in breast cancer. Therefore, this study has determined the level of TBX2 expression in tumor and non-tumor tissues in a cohort of breast cancer patients. Statistical analysis was performed to find the possible associations between TBX2 and clinical and pathological parameters. Furthermore, in this study, we determined the significance of TBX2 as a biomarker for breast cancer tissues.

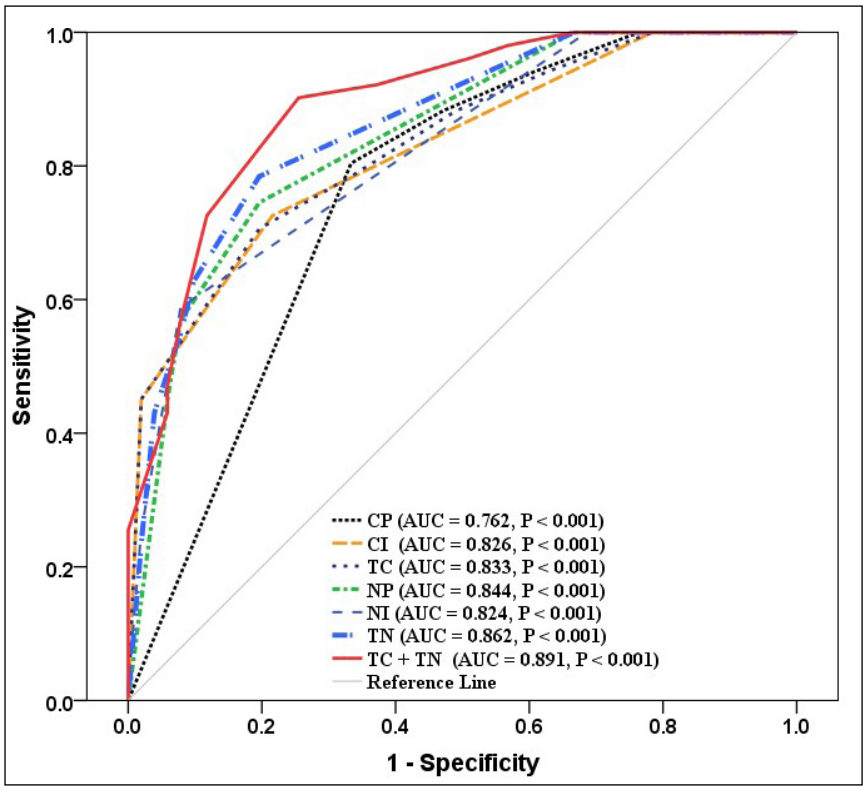

Fig. 2. ROC curve for cytoplasm and nuclear TBX2 as a biomarker to distinguish between tumor and non-tumor tissues. 95% CI; *p*-value < 0.05: Significant & *p*-value ≥ 0.05: Not significant.

Our results showed that TBX2 is significantly overexpressed in breast cancer tissues in comparison to non-tumor tissues. Importantly, the level of TBX2 was correlated with the lymph node size and metastasis which might indicate that TBX2 is overexpressed in breast cancer tissues of patients with high clinical stage. Clinical staging is one of the important parameters, which could judge the prognosis of cancer patients. Patients with a high clinical stage have a greater probability of distant metastasis and poor prognosis [34]. These findings are in agreement with several previous studies carried out on cervical, prostate, melanoma, ovarian, and breast cancers [9,17,20]. These observations suggest TBX2 overexpression as a promising biomarker for breast cancer cells with AUC of 0.891 (p<0.001).

CONCLUSIONS

In conclusion, the current study provides evidence that TBX2 is an overexpressed in significant percentage of breast cancer tissues and TBX2 significant positive correlations between TBX2 levels and lymph node size and metastasis. Immunohistochemical analysis showed that breast cancer tissues consistently show higher levels of TBX2 than non-tumor tissues, and these findings were confirmed by immunoblotting. Furthermore, the level of TBX2 was correlated with lymph node size and metastasis which suggests TBX2 as a useful diagnostic and prognostic marker for breast cancer.

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Ethical Committee:

The study was conducted according to Helsinki declaration and TWAS research grant award 2019.

Conflict of Interest:

The Authors declare that there is no conflict of interest.

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