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Original Article

T-box transcription factor 21 expression in breast cancer and its relationship with prognosis

Haiming Yu[1*], Junlan Yang[1*], Shunchang Jiao[1], Ying Li[1], Wei Zhang[2], Jiandong Wang[3]

[1]Department of Medical Oncology, General Hospital of PLA, Beijing, China; [2]Department of Pathology, 401 Hospital of PLA, Qingdao, China; [3]Department of General Surgery, General Hospital of PLA, Beijing, China. [*]Equal contributors.

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Abstract: Purpose: T-box transcription factor 21 (T-bet) is a key lineage-defining transcription factor. The purpose of this study was to verify the relationship between T-bet expression in primary tumors and prognosis of breast cancer. Methods: T-bet protein expression was immunohistochemically detected on surgically-obtained tumor samples of 130 (stage I-III) invasive breast carcinomas from Chinese subjects, who were followed up for a mean time of 112 months. Results: T-bet was expressed in the nuclei and cytoplasm of both tumor cells and tumor-infiltrating lymphocytes. In LOG-RANK analysis, higher density of interstitial T-bet+ interstitial lymphocytes was related with longer distant disease-free survival (DDFS) (P = 0.047); higher tumor nuclei T-bet expression was related with shorter DFS (P = 0.021) and DDFS (P = 0.026). Cox multivariate analysis showed that density of interstitial T-bet+ interstitial lymphocytes was an independent positive prognostic factor for DFS (HR = 0.474, P = 0.051) and DDFS (HR = 0.414, P = 0.030); tumor nuclei CTLA-4 expression was an independent adverse prognostic factor for DFS (HR = 3.007, P = 0.003), DDFS (HR = 2.931, P = 0.005) and OS (HR = 2.352, P = 0.029). Conclusions: This study found that, high tumor nuclei T-bet expression in primary tumors of breast cancer was correlated with poor prognosis and high density of T-bet+ interstitial lymphocytes in primary tumors of breast cancer were correlated with favorable prognosis.

Keywords: T-box transcription factor 21, breast cancer, disease-free survival, distant disease-free survival, overall survival

Introduction

T-bet, also known as T-box transcription factor 21, is a key lineage-defining transcription factor expressed in immune cells. T-bet plays critical roles in the differentiation of Th1 cells [1, 2], development of CD8+ effector cytolytic T lymphocytes [3, 4] and the cytotoxic activity of NK cells [5]. In dendritic cells, T-bet plays a pivotal part in the production of IFN-γ and priming of Th1 and cytotoxic T cells [3, 4]. All in all, T-bet expression in immune cells is indicative of active TH1-mediated immune responses. Immunohistochemical studies on breast cancer exhibited that infiltration of T-bet+ interstitial lymphocytes in primary tumor were correlated with better prognosis [6, 7]. Moreover, T-bet was found to be expressed in breast cancer cells [8] but the role of T-bet in tumor cells and its correlation with prognosis are poorly understood.

Materials and methods

Patients

The current study was retrospectively conducted in 130 patients who had undergone breast cancer surgery between January 2000 and December 2002 at the People's Liberation Army General Hospital, Beijing, China. The study was approved by the Institutional Review Board of the People's Liberation Army General Hospital. Informed consents were obtained from all the patients. The inclusion criteria include: (1) pathologically confirmed breast cancer, (2) availability of paraffin-embedded specimens of the primary tumor and relatively complete follow-up data. Out of 175 consecutive patients who had undergone radical mastectomy, 32 were excluded because of unavailability of primary tumor specimens and 13 cases were eliminated due to lack of follow-up data. Eventually, 130 patients were included.

Table 1. Clinicopathological features of 130 patients with breast cancer

Clinicopathological parameter		N (%)
Age (year)	≤ 48	66 (50.8)
	> 48	64 (49.2)
Menstrual status	Premenopausal	82 (63.1)
	Postmenopausal	48 (36.9)
Pathological type	Invasive ductal carcinoma	120 (92.3)
	Invasive lobular carcinoma	2 (1.5)
	Medullary carcinoma	3 (2.3)
	Mucinous carcinoma	2 (1.5)
	Invasive eczematous carcinoma of nipple	2 (1.5)
	Invasive ductal carcinoma and invasive lobular carcinoma	1 (0.8)
Tumor size	T1	47 (36.2)
	T2	70 (53.8)
	T3	11 (8.5)
	T4	2 (1.5)
Lymph node metastasis	N0	63 (48.5)
	N1	39 (30.0)
	N2	13 (10.0)
	N3	15 (11.5)
Clinical stage	I	31 (23.8)
	II A	42 (32.3)
	II B	24 (18.5)
	III A	16 (12.3)
	III B	2 (1.5)
	III C	15 (11.5)
SBR grading	I	17 (13.1)
	II	89 (68.5)
	III	24 (18.5)
Thrombosis	Positive	30 (23.1)
	Negative	100 (76.9)
ER	Positive	84 (64.6)
	Negative	46 (35.4)
PR	Positive	74 (56.9)
	Negative	56 (43.1)
HER-2	Positive	27 (20.8)
	Negative	103 (79.2)
Adjuvant chemotherapy	Yes	104 (80.0)
	No	26 (20.0)
Adjuvant radiotherapy	Yes	80 (61.5)
	No	50 (38.5)
Adjuvant endocrine therapy	Yes	71 (54.6)
	No	59 (45.4)

Immunohistochemistry

Serial paraffin-embedded sections (3 μm in thickness) from 130 patients were de-waxed with xylene and subsequently hydrated with an ethanol gradient. The tissue sections were subjected to high pressure in a Tris EDTA buffer (pH 9.0) for antigen retrieval. The tissue sections were immersed in 3% H_2O_2 for 10 minutes to eliminate endogenous peroxidase activity. The

working solution of normal goat serum was added to the tissue sections, and then incubated at 37°C in a humidified box for 10 minutes to block the non-specific antigens. The sections were then incubated overnight at 4°C with rabbit anti-human T-bet IgG (1:100 dilution, sc-21003, Santa Cruz, CA, USA). The slides were then incubated at 37°C for 30 minutes with a secondary antibody against rabbit and mouse immunoglobulins (Ready to use, EnVision K500711, DAKO, Denmark). Afterwards, the sections were stained with DAB for 1 minute. Nuclei were counter-stained with hematoxylin. The slides were then dehydrated with an ethanol gradient, mounted with neutral gum, and stored for later observation. Tissue sections obtained from biopsy specimens with confirmed high expression of the target molecules served as positive control, while incubation with the primary antibody diluent instead of the primary antibody were used as the negative control.

Imaging and data analysis

IHC slides were evaluated by two independent pathologists who were unaware of the clinical and prognostic information of the subjects. Three variables named density of T-bet+ interstitial lymphocytes, tumor nuclei T-bet expression and tumor cytoplasm T-bet expression were evaluated as follows: interstitial positive cells in interstitial adjacent to tumor nests were counted as follows: 15 high-power fields adjacent to tumor nests (400×) were randomly selected from the entire film under a LEICA DM2000 microscope. The interstitial positive cells per high-power field were counted and density of interstitial T-bet+ lymphocytes (average count of positive cells per square millimeter) was calculated by dividing positive cell count per high-power field area (0.31 mm^2). T-bet expression in tumor cell nuclei or cytoplasm was semi-quantitatively scored based on staining intensity: 1, light yellow; 2, brown; and 3, deep brownish yellow. The percentage of cumulative staining pattern of tumor cell nuclei or cytoplasm was sequentially drawn as A%, B%, and C%. Tumor nuclei T-bet expression or tumor cytoplasm T-bet expression (T-bet staining intensity in tumor cell nuclei or cytoplasm) was determined as (1 × A% + 2 × B% + 3 × C%).

Statistical analysis

Statistical analyses were performed using the SPSS 13.0 statistical software package. The correlation between continuous variables were assessed by using Spearman rank sum test; and correlation between categorical variables and clinicopathological parameters were evaluated by employing Mann-Whitney U test or Kruskal Wallis test. Non-parametric ROC analysis was utilized to determine the optimal cut-off values of variables for distant disease free survival (DDFS). The optimal cut-off values of density of T-bet+ interstitial lymphocytes for DDFS is 15.16/mm^2, the optimal cut-off values of tumor nuclei T-bet expression for DDFS is 0.13, the optimal cut-off values of tumor cytoplasm T-bet expression for DDFS is 0.55. The patients were divided into two groups, i.e., high- and low-expression group in terms of the optimal cut-off values of above three variables and were subjected to univariate and multivariate survival analysis, respectively. For survival analysis, the Kaplan-Meier method was used. For univariate analysis for significance test, Log-rank test or Cox analysis was employed. For multivariate analysis, the Cox proportional hazards model was utilized. A P value < 0.05 was considered to be statistically significant.

Results

Patient characteristics

The clinicohistopathological data of the enrolled subjects are summarized in **Table 1**. Pathological types included invasive ductal carcinoma (n = 120), invasive lobular carcinoma (n = 2), medullary carcinoma (n = 3), mucinous carcinoma (n = 2), invasive eczematous carcinoma of nipple (n = 2), invasive ductal carcinoma (n = 1) and invasive lobular carcinoma (n = 1). No patient received surgical castration, neoadjuvant chemotherapy and targeted therapy. The median follow-up time was 112 months (range: 7.7-138.6 months). Cases of local recurrence or metastasis were reported in 33 patients, whereas 29 patients died. The DFS, DDFS and OS rates were 74.6%, 76.2% and 77.7%, respectively. The median DFS, DDFS and OS could not be obtained.

T-bet expression in breast cancer

T-bet was expressed in cytoplasm and nuclei of tumor cells (**Figure 1A**) and interstitial lymphocytes (**Figure 1B**). 23 patients were negative for T-bet+ interstitial lymphocytes; and in the other patients, density of T-bet+ interstitial lymphocytes varied from 1.94/mm^2 to 93.33/mm^2. Tumor cell nuclei of 23 patients were T-bet neg-

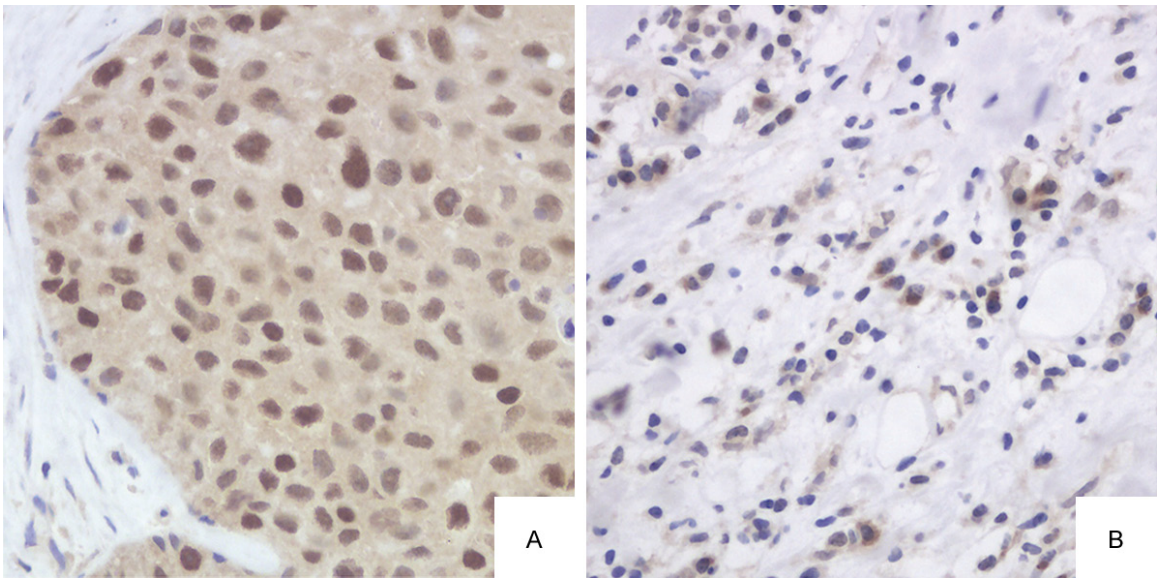

Figure 1. Expression patterns of T-bet in primary tumor of breast cancer. A. T-bet is expressed in cytoplasm and nuclei of tumor cells. B. T-bet is also expressed in cytoplasm and nuclei of interstitial lymphocytes.

Table 2. Univariate COX analysis of relationships between influencing factors and DFS, DDFS, and OS

Influencing factors	DFS		DDFS		OS	
	HR (95% CL)	*P* value	HR (95% CL)	*P* value	HR (95% CL)	*P* value
Clinical stage	2.599 (1.509-4.478)	0.001	2.827 (1.602-4.990)	< 0.001	3.156 (1.736-5.738)	< 0.001
Age	0.489 (0.237-1.009)	0.053	0.574 (0.275-1.198)	0.139	0.673 (0.317-1.427)	0.301
Menstrual status	0.995 (0.489-2.023)	0.988	1.165 (0.565-2.401)	0.675	1.315 (0.627-2.755)	0.469
ER	0.568 (0.286-1.128)	0.106	0.479 (0.236-0.971)	0.041	0.422 (0.203-0.879)	0.018
PR	0.473 (0.237-0.944)	0.034	0.440 (0.215-0.901)	0.025	0.368 (0.173-0.782)	0.009
HER-2	3.977 (1.990-7.947)	< 0.001	4.325 (2.128-8.790)	< 0.001	5.398 (2.601-11.200)	< 0.001
Thrombosis	2.680 (1.332-5.395)	0.006	2.568 (1.245-5.295)	0.011	2.993 (1.420-6.310)	0.004
SBR grading	3.204 (1.712-5.997)	< 0.001	3.633 (1.884-7.005)	< 0.001	3.904 (1.982-7.687)	< 0.001
Ki67	2.119 (1.042-4.311)	0.038	2.104 (1.008-4.394)	0.048	2.556 (1.163-5.617)	0.019
Adjuvant chemotherapy	1.115 (0.460-2.702)	0.809	1.018 (0.418-2.482)	0.968	0.923 (0.376-2.270)	0.862

ative; and in the other patients, T-bet expression in tumor nuclei ranged from 0.05 to 2.8. Tumor cell cytoplasm was T-bet negative in 16 patients and in the other patients, tumor cytoplasm T-bet expression changed in a range from 0.05 to 1.65.

Prognostic factors for DFS, DDFS and OS in breast cancer

Univariate analysis revealed significant associations between the clinical outcome and the established prognostic factors (age, clinical stage, SBR grade, tumor thrombi, ER and PR status, HER2 expression and Ki67), but failed to show any association between clinical outcome and adjuvant chemotherapy (**Table 2**).

T-bet expression and its correlation with clinicopathological features

Density of T-bet+ interstitial lymphocytes was not related to age, menopause, clinical stage, SBR grade, tumor thrombi, ER, PR, HER2 or Ki67. On the other hand, lower density of T-bet+ interstitial lymphocytes was associated with higher T stage (Mann-Whitney U, $P = 0.020$).

T-bet expression in tumor nuclei rather than tumor cytoplasm, was negatively correlated with age (Spearman, correlation coefficient = -0.182, $P = 0.038$). T-bet expression, in neither tumor nuclei nor tumor cytoplasm, was correlated to menopausal status, clinical stage, SBR grade, tumor thrombi, ER, PR, HER2, and Ki67.

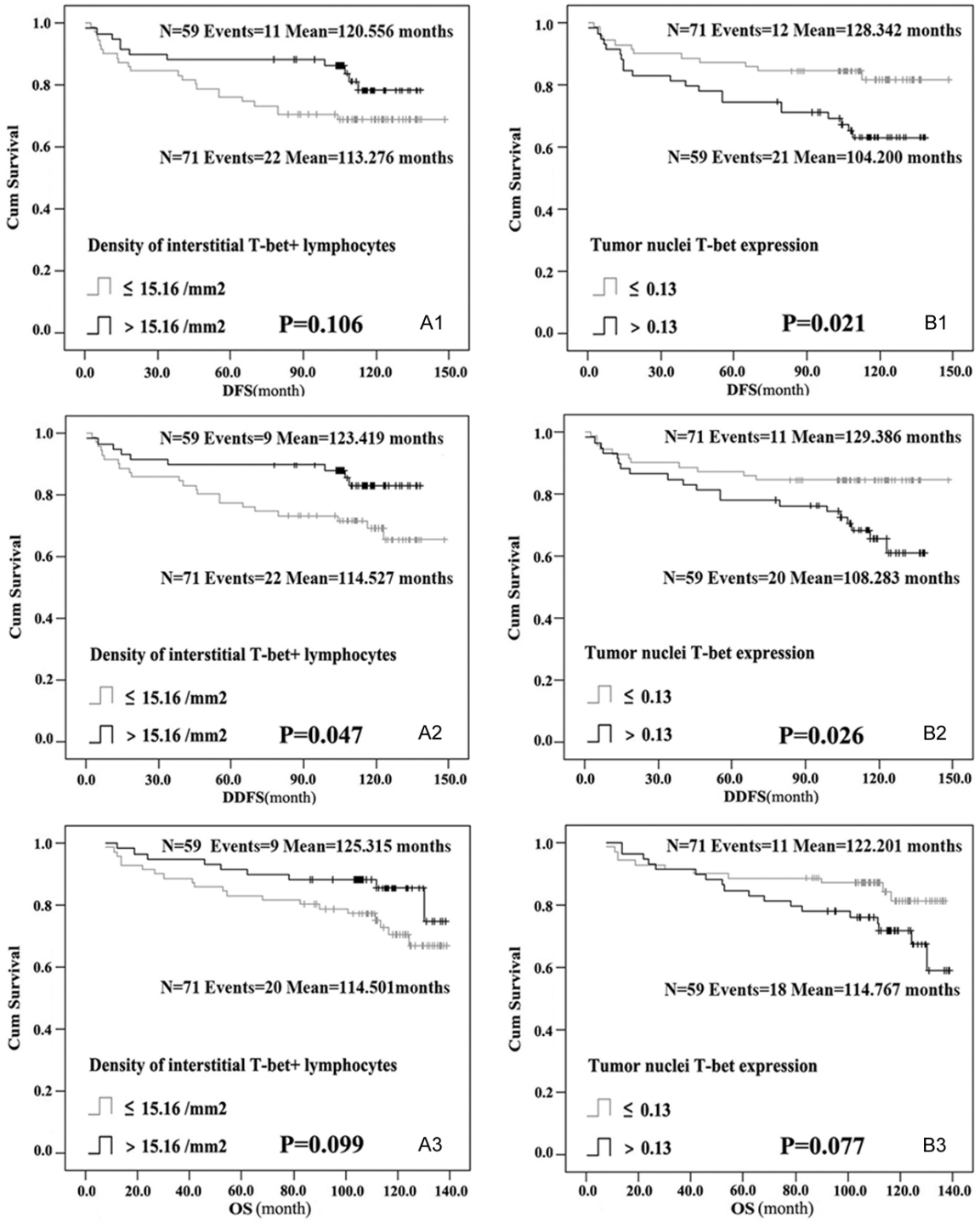

Figure 2. Prognostic value of T-bet expression in breast cancer. A. Patients were divided into low expression group and high expression group according to cut-off value (15.16/mm^2) of density of T-bet+ interstitial lymphocytes. Univariate Log-Rank analyses showed that high expression group had significantly longer DDFS (distant disease-free survival) than that of low expression group. There was no significant difference in DFS and OS between low expression group and high expression group. B. Patients were divided into low expression group and high expression group according to cut-off value (0.13) of tumor nuclei T-bet expression. Univariate Log-Rank analyses showed that low expression group had significantly longer DFS and DDFS than that of high expression group. There was no significant difference in OS between low expression group and high expression group.

Table 3. Multivariate COX analysis of relationships between influencing factors and DFS, DDFS, and OS

Survival	Influencing factors	HR (95% CL)	*P* value
DFS	Clinical stage	1.784 (1.022-3.111)	0.042
	SBR grading	2.197 (1.114-4.330)	0.023
	HER-2	3.389 (1.557-7.378)	0.002
	Density of T-bet+ interstitial lymphocytes	0.474 (0.224-1.002)	0.051
	Tumor nuclei T-bet expression	3.007 (1.446-6.255)	0.003
DDFS	Clinical stage	1.917 (1.055-3.481)	0.033
	SBR grading	2.525 (1.236-5.160)	0.011
	HER-2	3.318 (1.522-7.235)	0.003
	Density of T-bet+ interstitial lymphocytes	0.414 (0.186-0.919)	0.030
	Tumor nuclei T-bet expression	2.931 (1.380-6.226)	0.005
OS	Clinical stage	2.723 (1.484-4.996)	0.001
	SBR grading	2.146 (1.049-4.390)	0.037
	HER-2	4.143 (1.844-9.310)	0.001
	Tumor nuclei T-bet expression	2.352 (1.093-5.064)	0.029

Correlation between T-bet expression and DFS, DDFS or OS

LOG-RANK univariate analysis showed that higher density of T-bet+ interstitial lymphocytes was related with longer DDFS ($P = 0.047$); higher T-bet expression in tumor nuclei was related with shorter DFS ($P = 0.021$), DDFS ($P = 0.026$); T-bet expression in tumor cytoplasm was not related with DFS ($P = 0.500$), DDFS ($P = 0.646$) and OS ($P = 0.831$) (**Figure 2**). COX multivariate analysis exhibited that when controlled for age, menopausal status, clinical stage, SBR grade, tumor thrombus, ER, PR, HER2 and Ki-67, the density of T-bet+ interstitial lymphocytes was an independent favorable prognostic factor for DFS (HR = 0.474, 95% CL = 0.224-1.002, $P = 0.051$) and DDFS (HR = 0.414, 95% CL = 0.186-0.919, $P = 0.030$); CTLA-4 expression in tumor nuclei was an independent adverse prognostic factor for DFS (HR = 3.007, 95% CL = 1.446-6.255, $P = 0.003$), DDFS(HR = 2.931, 95% CL = 1.3806-6.226, $P = 0.005$) and OS (HR = 2.352, 95% CL = 1.093-5.064, $P = 0.029$) (**Table 3**).

T-bet expression in tamoxifen-resistant patients

Of 71 hormone receptor-positive patients who had received adjuvant endocrine therapy, 3 patients relapsed during the treatment and their tumors were taken as tamoxifen-resistant. T-bet expression level in tumor cell nuclei of the 3 patients were 0, 0.05 and 0.1, all being lower than 0.13 (the optimal cut-off values of tumor nuclei T-bet expression). So the 3 tamoxifen-resistant patients fell into the group of low T-bet expression in tumor cell nuclei.

Discussion

A previous research demonstrated that larger size of breast cancer was more closely associated with lower Th1/Th2 ratios in sentinel lymph nodes as compared with their small-sized counterparts [9]. The current study revealed that infiltration of T-bet+ interstitial lymphocyte was less intense as the tumor grew bigger. These findings collectively suggested that local immunosuppression aggravated as breast cancer progressed.

Chen et al. examined TILs isolated from fresh gastric cancer tissues by flow cytometry and found that T-bet+ TILs consisted of CD4+ T cells, CD8+ T cells and NK cells, with NK cells predominating among all cells [10]. Other researches found that active TH1-mediated immune response in tumor microenvironments was associated with favorable prognosis of breast cancer [11-13]. Consistent with these findings, the current study showed that higher density of T-bet+ TILs was correlated with longer DFS and DDFS in breast cancer.

Coincident previous results [8], our study exhibited that T-bet was extensively expressed in breast cancer cells. Though the roles of T-bet in immune cells have intensively studied, its part in breast cancer cells is poorly understood. T-bet, as a transcription factor, recognizes a conservative sequence termed the T-site in DNA to cause epigenetic changes: Changes in the chromatin environment of the target promoters of T-bet render it possible for the promoters to bind to other transcription factors [1]. The functional regulation of T-bet's target genes is cell context-dependent: Due to cell-type restricted cooperative factors, the expressions of T-bet target genes vary with different cell

types [1, 14]. Thus, T-bet, in breast cancer cells, may exert a regulatory effect different from that in immune cells. In a vitro experiment, reduced T-bet expression caused by T-bet siRNA treatment inhibited proliferation of MCF-7 cells upon insulin or E2 stimulation [8]. These findings suggested that T-bet is implicated in the proliferation of breast cancer cells. Therefore, in this study, high expression of T-bet in tumor nuclei was understandably correlated with poor prognosis of breast cancer.

In a previous study, breast cancer cell lines refractory to anti-estrogen therapy were found to express higher levels of T-bet than the cell lines sensitive to the anti-estrogen treatment, and MCF-7 cells over-expressing T-bet upon retrovirus packaging and transduction of T-Bet cDNA showed reduced sensitivity to tamoxifen in vitro [8]. Nonetheless, we failed to observe high T-bet expression in tumor cell nuclei in the three tamoxifen-resistant patients in this study. More studies are warranted to examine the relationship between T-bet expression and tamoxifen-resistance in breast cancer cells.

In summary, for the first time, this study demonstrated that that elevated T-bet expression in tumor cell nuclei was associated poor prognosis of breast cancer and high density of T-bet+ interstitial-lymphocytes was correlated with favorable prognosis of breast cancer. Our findings suggested that T-bet may play important roles in the progression of breast cancer cells. Further studies are needed to better understand the role of T-bet in the development of breast cancer.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Shunchang Jiao, Department of Medical Oncology, General Hospital of PLA, 28 Fuxing Road, Haidian District, Beijing 100853, China. Tel: +86-10-66939761; Fax: +86-10-66939761; E-mail: jiaosc@vip.sina.com

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