Original Paper

Higher Numbers of T-Bet⁺ Tumor-Infiltrating Lymphocytes Associate with Better Survival in Human Epithelial Ovarian Cancer

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Key Words

T-bet⁺ • Tumor-infiltrating lymphocytes • Epithelial ovarian cancer

Abstract

Background/Aims: T-bet, a member of the T-box family of transcription factors, is a key marker of type I immune response within the tumor microenvironment, and has been previously reported by us to serve as an important prognostic indicator for human gastric cancer patients and a potential biomarker for immunotherapy. In the present study, we aimed to assess the clinical significance and prognostic value of T-bet⁺ tumor-infiltrating lymphocytes in human epithelial ovarian cancer. **Methods:** The immunohistochemistry was used to analyze the infiltration density of T-bet⁺ lymphoid cells in human epithelial ovarian cancer tissues, and the flow cytometry analysis was used to further analyze the presence of T-bet⁺ tumor-infiltrating lymphocytes subgroups in cancer tissues. **Results:** Our immunohistochemistry analysis showed increased number of T-bet⁺ lymphoid cells in the human epithelial ovarian cancer tissues, and the flow cytometry analysis further demonstrated the presence of T-bet⁺ tumor-infiltrating lymphocytes subgroups including CD4⁺, CD8⁺ T cells and NK cells. In addition, we also observed a significant association of T-bet⁺ tumor-infiltrating lymphocytes density in the tumor nest of cancer with not only serum CA125 levels but also with distant metastasis. However no association was observed with other characteristics like patients' age, pathological type, FIGO stage, tumor site and tumor size. Furthermore, the survival analysis showed that higher density of T-bet⁺ tumor-infiltrating lymphocytes both in tumor nest and tumor stroma of cancer tissues was significantly associated with better patient survival. In addition, the density of T-bet⁺ tumor-infiltrating lymphocytes in tumor nest appeared to be an independent risk factor for predicting patients' postoperative prognoses. **Conclusions:** Our data indicated that the key transcription factor T-bet might play an important role in the

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type I immune cells mediated antitumor response, and the density of T-bet⁺ lymphocytes in human epithelial ovarian cancer tissues could serve as a prognostic predictor for ovarian cancer patients.

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Introduction

Ovarian cancer is the fifth leading cause of cancer-related deaths among women worldwide [1, 2], with epithelial ovarian cancer (EOC) as its major subtype. Most of the ovarian cancer patients usually have advanced disease at the time of diagnosis due to the asymptomatic nature of early-stage tumors, and thus result in poor long-term survival [3]. Although ovarian cancer is chemo-sensitive and generally show good initial response to platinum/taxane treatment, the 5-year recurrence rate still remains 60% to 80% [4].

It has been suggested that the tumor genesis and progression could induce adaptive immune responses, and the effective anti-tumor immunity appeared to be significantly associated with the cancer patients' prognoses [5-7]. A growing body of evidence have indicated that cell-mediated immune responses against the tumor tend to be critical component of tumor immune surveillance [8]. We have previously reported that the type I immune responses mediated by Th1 cells, CTLs, $\gamma\delta$ T cells, NK cells and NKT cells, are critical components of cell-mediated immunity against cancer [9-11]. T-bet, a hallmark transcription factor has been shown to be essential for the differentiation and function of these type I immune cells [5, 12]. Using animal models, the contribution of T-bet in anti-cancer immune responses, has been specifically attributed to its regulation of adaptive immune components like Th1 cells [13]. Numerous retrospective studies have also established that T-bet expression in human cancer tissues significantly associate with cancer progression and patients' prognoses [14-17]. In this regard, we have also previously reported that the higher numbers of infiltrating T-bet⁺ lymphocytes in human gastric and esophageal cancer tissues were significantly associated with the patients' postoperative prognoses [9, 10].

However, in the present study, we have focused on investigating the contribution of T-bet⁺ tumor-infiltrating lymphocytes (TILs) in human EOC tissues. To this effect, we have tried to analyze the density of T-bet⁺ TILs in EOC tissues and identify the association with patient's clinicopathological parameters and postoperative prognosis. Our study indicated that the tumor growth could elicit spontaneous type I cellular immune response and tumor progression appeared to be associated with suppression of antitumor immunity.

Patients and Methods

Patients

Formalin-fixed, paraffin-embedded EOC tissue samples were collected from 81 patients who underwent surgical resection between January 2007 and December 2012 in our hospital. None of the patients received pre-operative chemotherapy or radiotherapy. All the tumor tissues were confirmed as EOC after surgical resection by using hematoxylin and eosin (H&E) staining. The survival data were collected and the patient follow-up was performed until October 2015. The detailed clinical parameters of the patients, including age at diagnosis, FIGO stage [18], histological type classified according to the World Health Organization (WHO) criteria, tumor site, tumor size, preoperative CA125 levels and distant metastasis status were collected as shown in Table 1. The clinical information of some of the patients was not available or missing and thus they were excluded from the statistical analysis. In addition, twenty cases of benign ovarian cyst tissues resected from surgery were also collected and subjected to the immunohistochemistry (IHC) assay. Moreover, the fresh resected cancer tissues from three cases of ovarian cancer patients were used in the flow cytometry analysis to characterize the subgroups of T-bet positive lymphocytes in tumor infiltrating lymphocytes. This study was approved by the ethics committee of the Third Affiliated Hospital of Soochow University.

Table 1. Association between clinicopathological characteristics and the expression of T-bet⁺ TILs in tumor nest. Values in bold signify $P < 0.05$

Characteristics		Density of T-bet ⁺ TILs in tumor nest				χ^2	P
		Low		High			
		Cases	%	Cases	%		
Age (years)	< 50	18	69.23	8	30.77	0.878	0.645
	50 - 60	24	80.00	6	20.00		
	≥ 60	19	76.00	6	24.00		
Pathological type	Serous adenocarcinoma	44	81.48	10	18.52	3.32	0.068
	Others	17	62.96	10	37.04		
FIGO stage	I-II	9	56.25	7	43.75	3.606	0.058
	III-IV	50	79.37	13	20.63		
Tumor site	Unilateral	27	67.50	13	32.50	2.953	0.086
	Bilateral	32	84.21	6	15.79		
Tumor size	< 8cm	19	82.61	4	17.39	0.859	0.354
	≥ 8cm	40	72.73	15	27.27		
CA125 level	≤ 35	1	25.00	3	75.00	5.387	0.020
	> 35	57	77.03	17	22.97		
Distant metastasis	No	3	42.86	4	57.14	4.115	0.043
	Yes	56	77.78	16	22.22		

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues were cut into 3-μm-thick consecutive sections, and were dewaxed in xylene, rehydrated and graded in ethanol solutions. Antigen retrieval was performed by heating the tissue sections at 100°C for 30 min in citrate solution (10mmol/L, pH6.0). The sections were then immersed in a 0.3% hydrogen peroxide solution for 30 min to block endogenous peroxidase activity, rinsed in phosphate buffered saline (PBS) for 5 min, blocked with 3% BSA at room temperature for 30 min, and finally incubated with primary antibody against T-bet (dilution 1:120, Santa Cruz, USA) overnight at 4 °C. A negative control was performed by omitting the primary antibody. The sections were then incubated with horseradish peroxidase-labeled goat against mouse/rabbit secondary antibody (ready to use, Maixin Biotechnology Co., Ltd, Fuzhou, China). Sections were then dehydrated, cleared and mounted.

The density evaluation of T-bet⁺ TILs in ovarian tissues

All slides were examined independently by two pathologists who were blinded to patients' clinical data. The density of T-bet⁺ TILs in tumor nest was assessed as described in our previous publications [9, 10, 19]. In brief, numbers of T-bet⁺ TILs in tumor nest were determined according to the immunohistochemical staining and were counted as follows: five areas in tumor nest or in the tumor stroma with the most intense TILs were selected at low magnification (40x), and then the T-bet⁺ TILs were counted and recorded at high power field (HPF, 200x magnification). Similarly, the density of T-bet⁺ TILs in the tumor stroma was also assessed. Based on the density, the tissue samples were categorized as follows: Grade 0, scanty; Grade 1, moderate infiltration; Grade 2, abundant infiltration; Grade 3, massive infiltration. The group with Grade 0 and 1 constituted low infiltration group, while group with Grade 2 and 3 was defined as high infiltration group.

Intracellular staining and flow cytometry analysis

The TILs were harvested from fresh surgical resected ovarian cancer and benign ovarian cyst tissues according to the previously described methods [13]. Mouse anti-human CD45-PerCP, mouse anti-human CD4-APC-Cy7, mouse anti-human CD8α-PE-Cy7, mouse anti-human CD56-APC, mouse anti-human T-bet-PE antibodies, fluorescence labeled mouse or rat IgG Isotypes, and the FixPerm buffer were all purchased from eBioscience (San Diego, CA). The TILs were isolated from ovarian cancer and benign ovarian cyst by using the standard ficoll-hypaque density gradient centrifugation. For intracellular staining of T-bet, the TILs were surface stained, then permeabilized with the eBioscience FixPerm buffer. Subsequently, the cells were washed and stained with mouse anti-human T-bet-PE or mouse IgG1-PE Isotype Control. Flow cytometric analysis was performed using BD Canto II cytometer (BD Biosciences, San Jose, CA, USA), and the data was analyzed using FlowJo 10.0.6 software.

Statistical analyses

All data were analyzed with IBM SPSS statistical software (version 22.0, IBM Inc.). Quantitative data were expressed as mean ± standard deviation. The association between T-bet expression and the clinical

parameters was assessed with using chi-square test. Kaplan-Meier and Log-Rank test were used for comparing the different survival curves. The univariate and multivariate Cox model were used to estimate the association between T-bet expression and hazard risk of EOC patients. The *P* value less than 0.05 represented statistical significance.

Results

Immunolocalization of T-bet⁺ lymphocytes in ovarian tissues

To investigate the infiltration of T-bet⁺ lymphocytes in the ovarian tissues, we performed the immunohistochemical staining analysis of ovarian cancer and benign ovarian cyst tissues. As seen in Fig. 1, the positive T-bet staining signal was observed in the cell nuclei of the infiltrating immune cells in the ovarian cancer and benign ovarian cyst tissues. Based on the density of T-bet⁺ lymphocytes infiltration into the tumor nest of ovarian cancer tissues, we categorized the patients into two sub-groups: low infiltration, *n*=61, and high infiltration, *n*=20, with the cut-off value of 20 T-bet⁺ immune cells/HPF. In addition, the patients were also categorized into two sub-groups based on the density of T-bet⁺ immune cells infiltration into the tumor stroma of ovarian cancer tissues: without infiltration, *n*=13, and with infiltration, *n*=68.

Flow cytometry analysis of T-bet⁺ TILs subgroups in EOC tissues

In order to further study the subgroups of T-bet⁺ TILs in EOC tissues, we performed flow cytometry analysis of TILs isolated from fresh surgical resected EOC tissues from three cases of ovarian cancer patients. And as shown in Fig. 2, from one of the patients, we showed that T-bet could be detected in large portions of CD4⁺T cells, CD8⁺T cells and NK cells in the peripheral blood. In contrast, T-bet⁺CD4⁺T cells, T-bet⁺CD8⁺T cells and T-bet⁺ NK cells population comprised of 15.1%, 16.8% and 16.5% respectively among the TILs in EOC tissues.

The density of T-bet⁺ lymphocytes in ovarian cancer tissues and its relationship to patients' clinical parameters

Next, we tried to assess the correlation between the density of T-bet⁺ lymphocytes and patient clinical characteristics. Our analysis showed that T-bet⁺ lymphocytes density in the tumor nest of EOC tissues was significantly correlated with the serum CA125 levels (*P* =

Fig. 1. Positive T-bet immunostaining in the cell nuclei of the infiltrating immune cells in ovarian cancer as well as benign ovarian cyst tissues.

Fig. 2. Analysis of the peripheral blood displayed the percentages of T-bet⁺CD4⁺ T cells, T-bet⁺CD8⁺ T cells and T-bet⁺ NK cells as 32.0%, 50.8% and 94.9% respectively. Analysis of T-bet⁺CD4⁺ T cells, T-bet⁺CD8⁺ T cells and T-bet⁺ NK cells in the tumor tissue showed the percentage as 15.1%, 16.8% and 16.5%, respectively. NK cells were the largest group of cells among T-bet⁺ TILs.

Table 2. Association between clinicopathological characteristics and the expression of T-bet⁺ TILs in tumor stroma

Characteristics		T-bet ⁺ TILs in tumor stroma		χ^2	P
		Low	High		
		Cases	%	Cases	%
Age (years)	< 50	3	11.54	23	88.46
	50-60	6	20.00	24	80.00
	≥ 60	4	16.00	21	84.00
Pathological type	Serous adenocarcinoma	11	20.37	43	79.63
	Others	2	7.41	25	92.59
FIGO stage	I-II	1	6.25	15	93.75
	III-IV	12	19.05	51	80.95
Tumor site	Unilateral	6	15.00	34	85.00
	Bilateral	7	18.42	31	81.58
Tumor size	< 8cm	4	17.39	19	82.61
	≥ 8cm	9	16.36	46	83.64
CA125 level	≤ 35	0	0.00	4	100.00
	> 35	13	17.57	61	82.43
Distant metastasis	No	0	0.00	7	100.00
	Yes	13	18.06	59	81.94

0.020) and the distant metastasis ($P = 0.043$) as seen in Table 1. However, we did not find any significant correlation with patients' age, pathological type, FIGO stage, tumor site and tumor size ($P > 0.05$). Moreover, density of T-bet⁺ lymphocytes in the tumor stroma of EOC tissues and patients clinical parameters did not show any significant correlation ($P > 0.05$), as seen in Table 2.

Prognostic value of the density of T-bet⁺ TILs in EOC tissues

Finally we tested if the density of T-bet⁺ lymphocytes in EOC tissues has any prognostic value. Based on the immunostaining criteria, the 81 EOC patients were divided into two subgroups based on T-bet⁺ lymphocytes infiltration, namely high infiltration and low infiltration groups, as explained above, with cutoff value of 20 T-bet⁺ lymphocytes/ HPF. Survival analysis of the patients sub grouped on the basis of T-bet⁺ lymphocytes infiltration into the tumor nest of EOC, suggested that high infiltration group had significantly better patient

Fig. 3. The average OS times of patients with high or low expression of T-bet⁺ TILs in tumor nest were 71.4 and 49.8 months, respectively ($P = 0.009$), and in tumor stroma were 61.9 and 32.5 months, respectively ($P = 0.015$).

Table 3. Cox's proportional hazards model analysis of the density of infiltrating T-bet⁺ TILs in tumor nest. Values in bold signify $P < 0.05$

Characteristics	HR	Univariate 95% CI	P value	HR	Multivariate 95% CI	P value
Age (years)						
50 - 60/< 50	1.275	0.559-2.909	0.564	1.474	0.613-3.543	0.386
≥ 60/< 50	2.998	1.358-6.622	0.007	2.451	1.023-5.872	0.044
Pathological type						
Serous adenocarcinoma /others	1.035	0.533-2.011	0.92	1.537	0.729-3.24	0.259
FIGO stage						
III-IV/I-II	7.505	1.791-31.440	0.006	6.659	1.477-30.02	0.014
Tumor site						
Bilateral/unilateral	1.110	0.605-2.038	0.736	0.506	0.251-1.019	0.056
Tumor size (cm)						
≥ 8/< 8	0.582	0.302-1.122	0.106	0.798	0.385-1.652	0.543
T-bet ⁺ TILs in tumor nest						
High/low	0.276	0.098-0.780	0.015	0.235	0.07-0.795	0.020

Table 4. Cox's proportional hazards model analysis of the density of infiltrating T-bet⁺ TILs in tumor stroma. Values in bold signify $P < 0.05$

Characteristics	HR	Univariate 95% CI	P	HR	Multivariate 95% CI	P
Age (years)						
50 - 60/< 50	1.275	0.559-2.909	0.564	1.316	0.531-3.259	0.553
≥ 60/< 50	2.998	1.358-6.622	0.007	2.264	0.929-5.517	0.072
Pathological type						
Serous adenocarcinoma /others	1.035	0.533-2.011	0.92	1.224	0.589-2.544	0.588
FIGO stage						
III-IV/I-II	7.505	1.791-31.440	0.006	6.834	1.552-30.082	0.011
Tumor site						
Bilateral/unilateral	1.110	0.605-2.038	0.736	0.554	0.274-1.12	0.100
Tumor size (cm)						
≥ 8/< 8	0.582	0.302-1.122	0.106	0.651	0.316-1.341	0.244
T-bet ⁺ TILs in tumor stroma						
High/low	0.418	0.202-0.866	0.019	0.497	0.229-1.078	0.077

survival (Log-rank test, $P=0.009$), as shown in Fig. 3 (left). Similarly, survival analysis of EOC patients grouped based on the T-bet⁺ lymphocytes infiltration into the tumor stroma, also indicated that high infiltrating group had better survival (Log-rank test, $P=0.015$), as shown in Fig. 3 (middle). In addition, the multivariate analyses revealed that the density of infiltrating T-bet⁺ lymphocytes in tumor nest could be used as an independent risk factor for predicting patients' postoperative prognosis (HR=0.235, 95%CI:0.07-0.795, $P=0.020$, High vs. Low), as seen in Table 3. In contrast, the density of infiltrating T-bet⁺ lymphocytes in tumor stroma could not be used as an independent risk factor for predicting patients' postoperative prognoses (Table 4). Interestingly, the multivariate analyses showed that high density of infiltrating T-bet⁺ lymphocytes both in tumor nest and stroma had more power

Table 5. Cox's proportional hazards model analysis of the density of infiltrating T-bet⁺ TILs in tumor nest and stroma. Values in bold signify $P < 0.05$. #: Total patients were divided into 3 groups. Patients with the low density of infiltrating T-bet⁺ lymphocytes both in tumor nest and stroma were considered as group 1, named "Both Low". Patients with the high density of infiltrating T-bet⁺ lymphocytes only in tumor stroma were considered as group 2, named "Stroma High" (For there was no patient with the density of infiltrating T-bet⁺ lymphocytes only high in tumor nest in this study), and both high in tumor nest and stroma were considered as group 3, named "Both High"

Clinical parameters	HR	Univariate 95% CI	P value	HR	Multivariate 95% CI	P value
Age(year)						
50-60/<50	1.275	0.559-2.909	0.564	1.183	0.462-3.031	0.762
≥60/<50	2.998	1.358-6.622	0.007	2.417	0.989-5.906	0.053
Pathological type						
Serous adenocarcinoma/Others	1.035	0.533-2.011	0.92	1.482	0.690-3.186	0.313
Figo Stage						
III-IV/I-II	7.505	1.791-31.440	0.006	5.637	1.243-25.564	0.025
Location						
Bilateral/Unilateral	1.110	0.605-2.038	0.736	0.546	0.266-1.119	0.098
Tumor size(cm)						
≥8/<8	0.582	0.302-1.122	0.106	1.269	0.602-2.678	0.532
T-bet ⁺ TILs in tumor nest and stroma [#]						
Stroma High/ Both Low	0.540	0.256-1.143	0.107	0.636	0.284-1.427	0.272
Both High/ Both Low	0.180	0.056-0.577	0.004	0.180	0.047-0.694	0.013

in predicting patients' postoperative prognosis compared with patients with low density of infiltrating T-bet⁺ lymphocytes both in tumor nest and stroma (HR=0.180, 95%CI:0.047-0.694, $P=0.013$), as seen in Table 5.

Discussion

T-bet, an essential and functional modulator of Th1, CD8⁺T and NK cells activity, directly regulates the target gene IFN- γ [20]. We have previously reported that the infiltrating T-bet⁺ lymphocytes in human esophageal cancer and gastric cancer were significantly associated with cancer progression and patients' prognoses, and play an important role in the tumor immunosurveillance [9, 10, 21]. Moreover, we also characterized the subgroups of T-bet⁺ lymphocytes in gastric cancer tissues, and demonstrated that T-bet⁺ lymphocytes mainly consisted of CD4⁺T cells, CD8⁺T cells and CD56⁺NK cells [9]. Consistent with our publication, the study by Hennequin *et al.* also confirmed the correlation between increased infiltrating density of T-bet⁺ effector cells and better survival of human gastric cancer patients [15]. Similarly, the study by Mulligan *et al.* demonstrated that infiltrating density of T-bet⁺ lymphoid cells in breast cancer tissues was significantly associated with tumor size, high grade, hormone receptor negativity, CK5, EGFR and p53 status, high Ki-67, and basal subtype of the patients, and the reduced infiltrating density of T-bet⁺ lymphoid cells was significantly associated with the reduced disease-free survival of the patients [22].

Here in this study, we have examined the infiltration of T-bet⁺ lymphocytes by their immunostaining in ovarian cancer as well as benign ovarian cyst tissues. In addition, the flow cytometry analysis further demonstrated the presence of T-bet⁺ lymphocytes subgroups including CD4⁺T cells, CD8⁺T cells and NK cells in EOC tissues. Due to the limitation of the number of ovarian cancer patients involved in the flow analysis, we shall further investigate the correlation of the percentages of T-bet⁺ lymphocytes subgroups and the patients' pathological parameters as well as patients' prognoses in future study. In the present study, our immunohistochemistry data showed that the infiltrating density of T-bet⁺ lymphocytes in the tumor nest of EOC tissues was significantly correlated with the serum CA125 levels and the distant metastasis, but not with patient's age, pathological type, FIGO stage, tumor

site and tumor size. Moreover, the survival analysis indicated that the higher density of T-bet⁺ lymphocytes both in tumor nest and stroma of the EOC tissues was significantly associated with better survival of the patients. Moreover, the density of infiltrating T-bet⁺ lymphocytes in tumor nest appeared to be an independent risk factor for predicting patients' postoperative prognoses. Thus, our data indicates that the transcript factor T-bet plays an important role in the type I immune cells mediated antitumor response during ovarian cancer progression and the density of T-bet⁺ lymphocytes in EOC tissues could be used as a prognostic predictor.

In addition, we have previously reported that T-bet not only control the adaptive anti-tumor immune response in the tumor microenvironment by promoting Th1 fate and suppressing alternative Th2 and Th17 differentiation, but could also promote the T cell trafficking into the tumor sites via up-regulating CXCR3 [13]. Another independent study also showed that T-bet in combination with Eomes could control the development and survival of the tissue resident memory T cells via regulating cytokines TGF- β and IL-15, and thus contributes in the protection against infections [23]. T-bet has also been linked with the regulation of NK cell biology [24]. Study by Hamilton *et al.* demonstrated that T-bet in companion with ZEB2 contributed critically for the generation and expansion of terminally differentiated T cells and NK cells, thereby suggesting the role of these key transcription factors in controlling the differentiation and functional prowess of these effector cells [25].

In conclusion, our current study demonstrated that the density of infiltrating T-bet⁺ lymphoid cells in human EOC tissues could serve as an important prognostic predictor. In addition, we also observed that T-bet can hold a great promise for the effective treatment against human malignancies including EOC, due to its active role in antitumor immune responses.

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Disclosure Statement

The authors disclose no potential conflicts of interest.

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