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Evaluation of PTEN and CD4$^+$FOXP3$^+$ T cell expressions as diagnostic and predictive factors in endometrial cancer

A case control study

Zeng Xi, MD[a,b], Li Jing, MD[c], Kang Le-Ni, MD[b,d], Lan Zhu, MD[a,b], Deng Ze-Wen, MD[a,b], Ye Hui, MD[a,b], Xi Ming-Rong, MD[a,b], Liao Guang-Dong, MD[a,b,*]

Abstract

To evaluate the potential role of Pten and CD4$^+$FOXP3$^+$ T cells in prognosis from endometrial cancer.

Tissue samples and clinical data were collected from 200 patients with endometrial cancer and 100 control patients with benign uterine diseases. The expressions of Pten and CD4$^+$FOXP3$^+$ T cells were quantified by immunohistochemistry and immunofluorescence. After surgery, all patients were followed up for an average of 56.3 months. Surgical effects were evaluated based on the patients' symptoms and signs. A two-sided P value $< .05$ was considered significant.

Pten diminished and CD4$^+$FOXP3$^+$ T cells significantly accumulated with the progression of endometial cancer, in comparison to the controls. Moreover, Pten expression was negatively correlated with the count of CD4$^+$FOXP3$^+$ T cells. Pten and CD4$^+$FOXP3$^+$ T cells were correlated with clinical characteristics, including tumor stage, differentiation and associated with patients' disease-free survival.

Limited data were available between the expressions of Pten and CD4$^+$FOXP3$^+$ T cells in patients with endometrial cancer. Our study findings suggested that the expressions of Pten and CD4$^+$FOXP3$^+$ T cells might become possible biomarkers for the diagnosis and prediction in endometrial cancer.

Abbreviations: BMI = body mass index, CA-125 = carbohydrate antigen 125, CA19-9 = carbohydrate antigen 19-9, CEA = carcinoembryonic antigen, DFS = disease-free survival.

Keywords: CD4$^+$FOXP3$^+$T cell, diagnosis and predictive, endometrial cancer, Pten

1. Introduction

Endometrial cancer (EC) is the second most common cancer among females worldwide. It is more frequent in the developed countries than that in the developing countries, with an incidence of 1.6% and of 0.6%, respectively,[1] with more than 61,380 new cases diagnosed in America in the year of 2017.[2] However, there is no effective way to early detection.

Nowadays, the early diagnosis of EC is generally based on the clinical manifestation such as postmenopausal bleeding or abnormal serum levels of certain tumor markers. While, about 15% of ECs occur in women without vaginal bleeding.[3] Previous literatures reported the role of different serum markers in the diagnosis of endometrial cancer such as the carcinoembryonic antigen (CEA), the carbohydrate antigen-125 (CA-125), and the carbohydrate antigen 19-9 (CA19-9), but these markers were elevated in only 20% to 30% of patients.[3,4]

In recent years, with the development of gene sequencing, the roles of gene regulation and the microenvironment in the development of carcinogenesis have been focused and better understood. It is highly potential that some gene markers might also be related to the development of ECs that could be promising in the diagnosis especially early diagnosis of ECs.

Pten, also known as the late tumor mutated gene, locating at 10q23.3, is 200 kb in length, spans 9 exons and 8 introns, and was discovered by researchers from the Columbia University in 1997.[5] Pten maintains normal cellular activities by negatively regulating multiple signal transduction pathways, inhibiting the proliferation and migration of tumor cells, and inducing apoptosis of tumor cells, and play a key role in the development of immune response. Accordingly, Pten mutations have been found in various cancers.[6–12] Indeed, Pten tends to become gradually silenced as lung, prostate, colon, and bladder cancers progress.[12–16] In addition, even a slight reduction in Pten expression has a major impact on cancer susceptibility in animal models.[17] Previous studies also found that about 20% of patients had endometrial atypical hyperplasia harbor Pten

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[a] Department of Gynecology and Obstetrics, The West China Second University Hospital, Sichuan University, [b] Key Laboratory of Birth Defects and Related Diseases of Women and Children (Sichuan University), Ministry of Education, Chengdu, [c] Department of Environmental and Occupational Health, West China School of Public Health, Sichuan University, Chengdu, Sichuan, [d] National Office for Maternal and Child Health Surveillance of China, West China Second University Hospital, Sichuan University, Chengdu, Sichuan, China.

[*] Correspondence: Liao Guang-Dong, China (e-mail: liaoguangdong2018@126.com).

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mutations, indicating that the loss of Pten might be an early event in the development of endometrial cancer. Animal experiment also found that $Pten^{+/-}$ mice develop endometrial atypical hyperplasia, of which approximately 20% progress to highly differentiated cancer.[18]

Immune tolerance is an important phenomenon as a specific non-response or low-response state when immunocompetent cells encounter antigenic substances. Accordingly, this state enables tumor cells escape from the immune surveillance, and limits the effectiveness of tumor immunotherapy. Regulatory T cells, which was firstly described by Gershon and Kondo in the early 1970s,[19,20] are now known to induce and maintain immune tolerance. These cells are sub-divided into $CD4^+$ and $CD8^+$ subtypes based on the surface markers, secreted cytokines, and mechanisms of action. $CD4^+CD25^+FOXP3^+$ T cells (Treg cells) derived from the thymus are among the most widely studied T cell populations.[21]

$CD4^+FOXP3^+$ T cells in the peripheral blood were reported to be more abundant in patients with breast cancer, colorectal cancer, esophageal cancer, gastric cancer, hepatocellular carcinoma, leukemia, lung cancer, lymphoma, melanoma, ovarian cancer, and pancreatic cancer. These peripheral $CD4^+FOXP3^+$ T cells strongly inhibit T cells *in vitro*, and hence may suppress immune activity against tumor-associated antigens. Indeed, tumors repress immune activity against through transfer, differentiation, and proliferation of Treg cells, enabling escape from immune surveillance and thus continued growth and development.[22] For example, the accumulation of $CD4^+FOXP3^+$ T cells in the tumor microenvironment and the abundant expression of FOXP3 promote ovarian cancer development in chimeric animal models with severe combined immunodeficiency, presumably by inhibiting cytotoxic $CD8^+$ T cells that target tumor-associated antigens. The accumulation of $CD4^+FOXP3^+$ T cells in tumor tissues is also significantly associated with poor prognosis.[23] Conversely, conditional knockout of $CD4^+FOXP3^+$ T cells was found to impede the progress of melanoma and colon cancer in mice, as well as to elicit massive apoptosis in metastatic breast cancer cells.[24–26]

Recently, it has been revealed that PTEN can increase Treg stability and that the loss of PTEN can lead to spontaneous inflammatory disorder.[27] The PI3K/AKT/mammalian target of rapamycin (mTOR) signaling pathway is pivotal for Treg cell development and homeostasis.[27–33] And PTEN is one of the pivotal controler in PI3K/AKT/mTOR signaling pathway, which can present in the microclusters of Treg cells and modulate the AKT phosphorylation status in immune synapses in a coordinated manner.[34] This pathway is activated downstream of the T-cell receptor (TCR), CD28, and IL-2 signaling. It is critically involved in Treg cell thymic development, peripheral expansion, and suppressive activity.[33] These observations highlight PTEN's importance of a stringent negative regulation of PI3K pathway activity in Treg cells.[34]

Based on the above pathway, Pten mutations have also been detected in the T cell-associated malignancies. For example, mice with specific Pten mutations exhibit increased B cell proliferation and serum autoantibodies as well as enhanced cell migration, proliferation, and resistance to apoptosis.[35,36] In addition, an enhanced proliferation of $CD4^+FOXP3^+$ T cells, a phenotype rescued by Pten re-expression, was also shown in the Pten-deficient mice.[37] Similarly, functional Pten was found to be essential to maintain the homeostasis of $CD4^+FOXP3^+$ T cells and thus of the immune system.[27,28]

However, the relationship between the Pten expression and the $CD4^+FOXP3^+$ T cells in patients with endometrial cancer has been poorly characterized. A hospital based case control study was conducted to explore the possible correlation.

2. Materials and methods

2.1. Patients and controls

Approval from the ethics committee of West China Second University Hospital, Sichuan University, China, was obtained at the beginning of the project. Two hundred patients with endometrial cancer were recruited on admission to the hospital from January 2014 to January 2015. These patients were diagnosed by curettage of uterus, and each diagnosis was confirmed by two pathologists. Tumors were staged according to the 2009 criteria for adenocarcinoma of the endometrium, as defined by the International Federation of Gynecology and Obstetrics.

For the case group, inclusion criteria were:

1. pre-operative pathological diagnosis of endometrial cancer;
2. no chemotherapy or radiotherapy;
3. no hormone therapy;
4. good compliance.

The exclusion criteria were:

1. women with a previous history of tumors;
2. with chemotherapy or radiotherapy history;
3. with hormone therapy history;
4. patients with severe internal and surgical diseases.

Another 100 age-matched females, who underwent hysterectomy at the hospital due to benign uterine diseases, and without history of cancers during the same time period, were recruited as controls. Written informed consent was obtained from all patients, in compliance with the Declaration of Helsinki.

2.2. Sectioning of tissues

Endometrium tissues were collected during surgery, and sectioned at 4 μm. Clinical and histological data were collected.

2.3. Immunohistochemistry for Pten

As part of routine clinical practice, three samples of primary tissue per patient were randomly selected and assayed for Pten by conducting immunohistochemistry (Abcam, USA). Staining pattern, intensity, and distribution were evaluated by using light microscopy. Pten was considered present if distinct brownish nuclear staining was observed. Stained mononuclear cells were reported as percentage of all immune cells in the field.

2.4. Immunofluorescence for $CD4^+FOXP3^+$T cells

Three samples of primary tissue per patient were randomly selected. Paraffin-embedded slides were deparaffinized, rehydrated, microwaved in the EDTA pH 9.1 to retrieve antigens, and were then stained for $CD4^+FOXP3^+$ T cells by using the fluorescently labeled rabbit anti-human CD4 (Abcam, USA) and the mouse anti-human FOXP3 (Abcam, USA). Slides were imaged under a fluorescence microscope. Images obtained at different wavelengths were superimposed in Image Processing

and Analysis in Image J (National Institute of Health, USA) to identify cells with fluorescently stained cell membranes, nuclei, or cytoplasm. $CD4^+FOXP3^+$ T cells were quantified in each sample as (cumulative optical density after correction of CD4 fluorescence index + cumulative optical density after correction of FOXP3 fluorescence index)/2.[38]

2.5. Statistical analysis

Data were analyzed by using SPSS version 22.0 (IBM Company, USA) and are reported as mean ± standard deviation and range. Categorical variables were compared between patients and controls by using Chi-squared test. After testing for normality of the raw data, the correlation between clinical factors and levels of Pten and Treg cells was analyzed by using student *t*-test. A two-sided P value $< .05$ was considered significant. Figures were generated in GraphPad Prism 5 (GraphPad Software Inc., USA).

3. Results

As listed in Table 1, cases and controls have comparable clinical characteristics, with P values $> .05$.

3.1. Pten expression in endometrial tissues

Abnormal proliferation of glandular tissues, structural disorder, heterotypic cells, large nuclei, irregular morphology, enhanced mitosis, less differentiation, smaller glandular structures, and obvious lymphocyte infiltration were observed in endometrial tissues collected from patients, as assessed by immunohistochemistry and microscopy (Fig. 1).

Table 1

Clinical characteristics of patients and controls.

Characteristics	Patients, n (%)	Controls, n (%)	*P* value
Sample size	200	100	
Mean age ± SD (range), years	56.8±9.78 (31–78)	55.03±7.41 (29–74)	.833
BMI mean ± SD, kg/m^2	22.17±1.76	22.98±2.21	.898
Family history of cancer			
Yes	41 (20.5%)	17 (17%)	.469
No	159 (79.5%)	83 (83%)	
Menopausal status			
Premenopausal	112 (56%)	55 (55%)	.869
Postmenopausal	88 (44%)	45 (45%)	
History of pregnancy			
Yes	187 (93.5%)	92 (92%)	.631
No	13 (6.5%)	8 (8%)	
Uterine bleeding			
Yes	161 (80.5%)	No data	
No	39 (19.5%)	No data	
FIGO stage			
I	158 (79%)	0	
II	34 (17%)	0	
III	8 (4%)	0	
IV	0 (0%)	0	
Differentiation stage			
G1	69 (34.5%)	0	
G2	77 (38.5%)	0	
G3	54 (27%)	0	
Histology			
Adenocarcinoma	160 (100%)	0	
Non-adenocarcinoma	0 (0%)	0	

Pten expression was the highest in normal endometrial cells, and was the lowest in G3 endometrial cancers, with an average staining score of 6.6 points for the control tissues, however, of 4.4 points for the tumor tissues. The average staining scores for highly differentiated (Grade 1, G1), moderately differentiated (Grade 2, G2), and poorly differentiated (Grade 3, G3) tissues were 5.9, 3.5, and 1.7 points, respectively. Tumors with staining score < 4.4 were considered as weakly expressing Pten (98/200 cases, 49%), while those with staining score ≥ 4.4 were considered as strongly expressing (102/200 cases, 51%). Remarkably, we found that Pten staining intensity was associated with diabetes in cancer patients, and with the clinical stage and differentiation grade of the tumor (Table 2).

Similarly, control tissues with staining score < 6.6 were considered as weakly expressing (44/100 cases, 44%), while those with staining score ≥ 6.6 were considered as strongly expressing (56/100 cases, 56%). However, we found that Pten staining intensity was not significantly associated with menopause, obesity, diabetes, and hypertension (Table 3).

3.2. Relationship between Pten expression and diagnosis

Furthermore, we analyzed the ROC of Pten expressions and the AUC was 0.839, 95% CI:0.795–0.884. The best cut-off value was 4.75 (Fig. 2). Based on the value, the specificity was 88%, and sensitivity was 65.5%.

3.3. Relationship between Pten expression and prognosis

Excluding 11 patients (3 with weak and 8 with strong expression) who were lost during follow-up, the average disease-free survival (DFS) was 54.53 months and 58.6 months ($P = .048$) for patients with tumors weakly (97 cases) and strongly (92 cases) expressing Pten.

For patients with weakly infiltrated tumors, 23 cases were recurrenced. The earliest and the latest recurrence times were 5 and 55 months after surgery, respectively, with the median of 29.9 months. Detailed, for patients with strongly infiltrated tissues, 10 cases relapse occurred between 17 and 55 months after surgery with the median time to recurrence of 36.5 months (Fig. 3). Univariate statistical analysis also showed that Pten expression was statistically associated with postoperative recurrence (Table 4).

3.4. Accumulation of $CD4^+FOXP3^+$ T cells in endometrial tissues

Immunofluorescence staining showed that $CD4^+FOXP3^+$ T cells were less abundant in normal endometrial tissues than that in endometrial carcinomas, with average staining scores of 0.7 and 3.3, respectively. Accordingly, tumors with staining score < 3.3 were considered as weakly infiltrated with $CD4^+FOXP3^+$ T cells (117 cases, 58.5%), while those with staining score ≥ 3.3 were considered as strongly infiltrated (93 cases, 46.5%). Staining scores for highly, moderately, and poorly differentiated were 1.2, 2.8, and 4.7 points, respectively ($P = .015$) (Fig. 4). We found that infiltration of $CD4^+FOXP3^+$ T cells was related to the presence or absence of BMI, obesity, as well as to tumor differentiation (Table 5). However, there was no significant relationship between $CD4^+FOXP3^+$ T cells and the clinical data in the controls (Table 6).

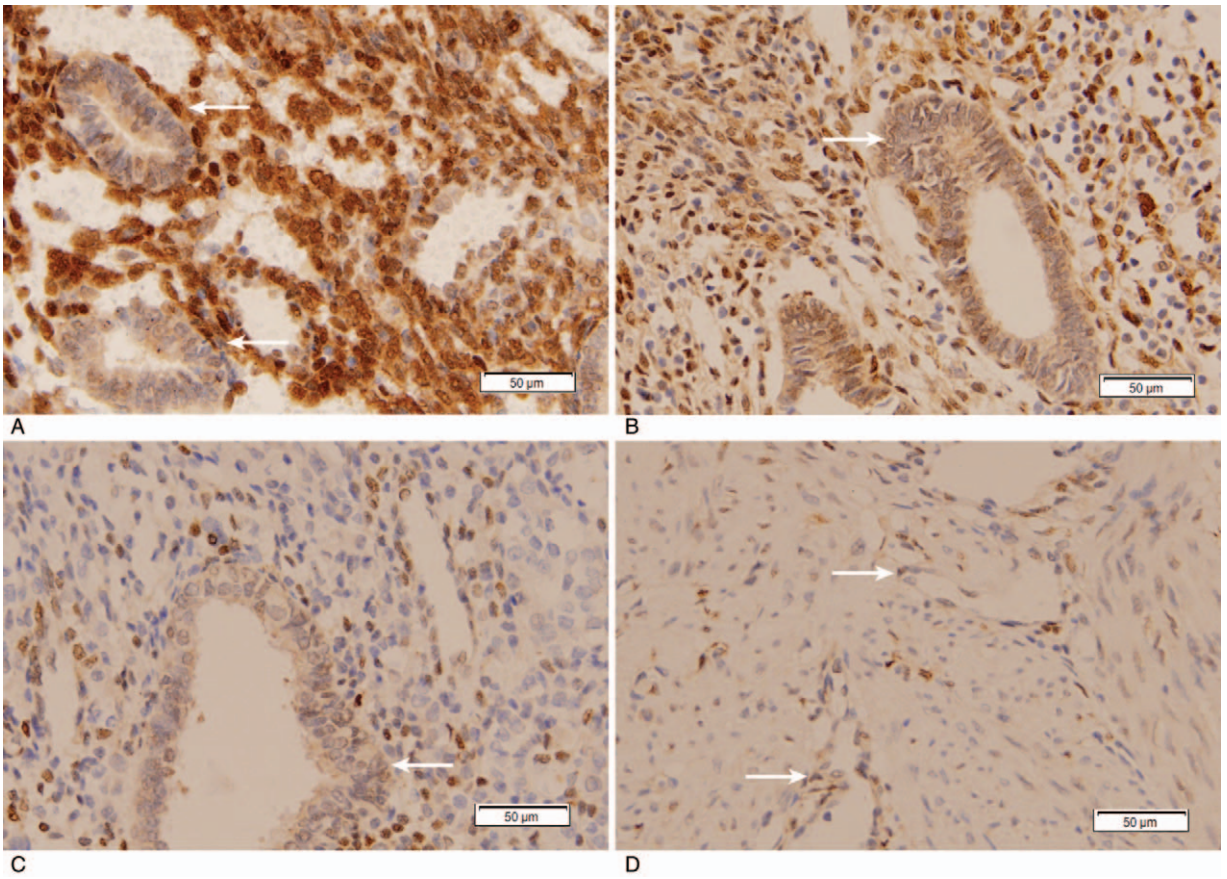

Figure 1. Immunohistochemical staining expression of Pten in endometrial normal tissues and adenocarcinoma of all grades. (A) Normal, ×20, *ARROW*: normal nuclei and glandular tissue structure; (B) highly differentiated, ×20, *ARROW*: approximately normal glandular structure but with enhanced mitosis and large nuclei; (C) moderately differentiated, ×20, *ARROW*: large nuclei, structural disorder of glandular tissues; (D) Poorly differentiated, ×20, *ARROW*: structural disorder and irregular morphology of glandular tissues, heterotypic cells.

Table 2

Association between Pten expression and clinical characteristics in patients.

Characteristics	Number (N = 200)	Pten expression		*P* value
		Weak (n = 98)	Strong (n = 102)	
Menopausal status				
Premenopausal	112	55	57	.973
Postmenopausal	88	43	45	
BMI				
< 28	118	63	55	.136
⩾28	82	35	47	
Diabetes				
Yes	32	21	11	.015
No	168	71	97	
Hypertension				
Yes	47	30	17	.216
No	153	82	71	
FIGO stage				
I	158	69	89	.017
II/III	42	27	15	
Differentiation				
I	69	23	46	.004
II	77	46	31	
III	54	29	25	

3.5. Relationship between CD4⁺FOXP3⁺ T cells expression and diagnosis

The ROC of Pten expressions and the AUC was 0.759, 95% CI: 0.687–0.831. The best cut-off value was 1.15 (Fig. 5). Based on the value, the specificity was 78%, and sensitivity was 83.5%.

Table 3

Association between Pten expression and clinical characteristics in control volunteers.

Characteristics	Number (N = 100)	Pten expression		*P* value
		Weak (n = 44)	Strong (n = 56)	
Menopausal status				
Premenopausal	59	23	26	.686
Postmenopausal	41	21	20	
BMI				
< 28	67	32	35	.280
⩾ 28	33	12	21	
Diabetes				
Yes	17	11	6	.059
No	83	32	51	
Hypertension				
Yes	12	8	4	.124*
No	88	36	52	

* By Fisher exact probability method.

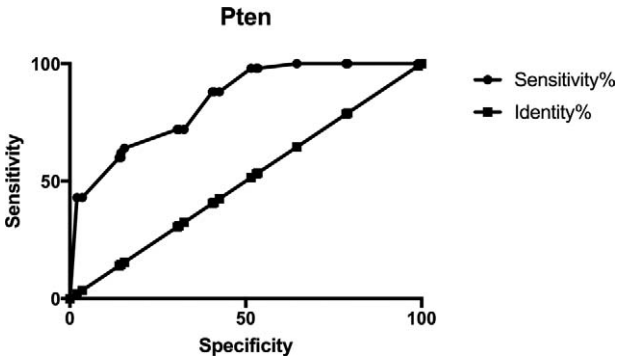

Figure 2. The ROC curves of Pten expressions among the subjects.

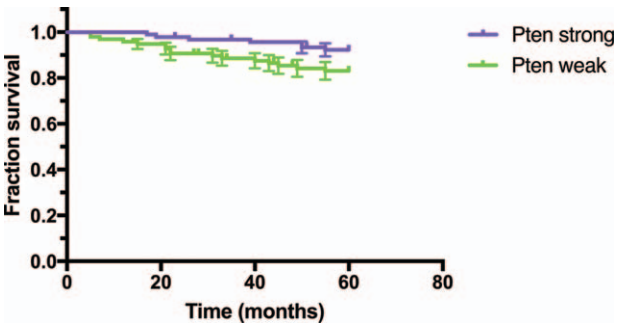

Figure 3. Relationship between Pten expression and prognosis into tumors.

3.6. Relationship between prognosis and abundance of $CD4^+FOXP3^+$ T cells

Eleven patients (10 in week and 1 in strong) were excluded for final analysis due to the lost of follow up.

The average follow-up time for 97 patients with weakly infiltrated tumors was 59.6 months. Among them, 10 cases suffered recurrence, and the earliest and latest recurrence times were 17 and 55 months after surgery, respectively, with a median of 37.6 months. The mean DFS was 58.42 months (Fig. 6).

The average follow-up time for 92 patients with strongly infiltrated tissues was 52.78 months. Twenty-two relapse cases occurred between 5 and 55 months after surgery. The median time to recurrence was 27.1 months. Mean DFS was 54.53 months ($P = .044$, Fig. 4). Univariate statistical analysis showed that $CD4^+FOXP3^+$ T cell infiltration was statistically associated with postoperative recurrence (Table 7).

3.7. Correlation between Pten and $CD4^+FOXP3^+$ T cell expression

Stainings for Pten and $CD4^+FOXP3^+$ T cell were normally distributed in the controls, highly, moderately, and poorly differentiated tissues. Pearson correlation analysis showed that Pten expression was negatively correlated with $CD4^+FOXP3^+$ T cell expression in all groups (Fig. 7).

4. Discussion

In this study, we analyzed the expression of Pten and $CD4^+FOXP3^+$ T cell among 200 patients with endometrial cancer who were treated in our hospital, and another 100 controls who underwent hysterectomy for benign gynecological diseases. There was no significant difference between the two groups in terms of age of disease onset, body mass index, family history of cancers, menopausal status, and gestational age.

The Pten mutations and deletions are unremarkable in esophageal cancers, although Pten expression at mRNA or protein level is exceptionally low, implying that defects may happen during the transcription and the translation.[17] Gabriel et al also reported that Pten deletion in prostate cancers was predictive to the tumor stage and grade.[12] However, among the endometrial diseases, Mutter et al found that Pten mutations were present in about 20% of patients with endometrial atypical hyperplasia, and in about 83% of patients with endometrial cancer. As Pten mutations are normally absent in the healthy population, suggesting that loss of Pten may be an early event in the process of endometrial carcinogenesis.[39] In addition, Konopka et al reported that the severity and nature of Pten mutations in endometrial cancer were closely related to the tissue localization and their clinical features.[40] Janiec et al also found that Pten mutations in endometrial cancer is positively related to its differentiation level, the more Pten mutations, the higher differentiation level. Also, the Pten mutation was found more common in patients < 55 years than that in the older patients.[41] Our study had consistent findings with the previous studies that the average Pten expression was the highest in the normal endometrial tissues, and negatively correlated with tumor grade in endometrial adenocarcinoma, association with the clinical stage, FIGO stage and differentiation grade of the tumor.

$CD4^+FOXP3^+$ T cells are important mediators for tumor immune escape, and are currently considered the most representative of immunoregulatory $CD4^+$ T cells. Importantly, immune escape is associated with prognosis from cancer. For example, levels of $CD4^+FOXP3^+$ T cells in peripheral blood were significantly enhanced in patients with breast cancer, colorectal cancer, esophageal cancer, gastric cancer, hepatocellular carcinoma, leukemia, lung cancer, lymphoma, melanoma, ovarian cancer, and pancreatic cancer. These cells may inhibit immune responses to tumor-associated antigens.[23,26,42–45] In addition, mice with lymphoma and specific Pten mutations in T cells showed increased $CD4^+$ T cell proliferation, elevated serum autoantibodies, and enhanced lymphoma cell migration and proliferation.[19,24,46] In this present, we also found that levels of $CD4^+FOXP3^+$ T cells in endometrial adenocarcinomas were significantly increased. The increased levels of these cells suggested later tumor stage and more severe grade, which indicates worse prognosis. Correlation analysis confirmed that Pten expression was negatively correlated with $CD4^+FOXP3^+$ T cell expression. Indeed, Pten expression decreased with tumor grade, normally accompanied with $CD4^+FOXP3^+$ T cells accumulated.

Diabetes is a recognized risk factor that promotes the development of endometrial cancer.[47–49] Our studies also have

Table 4

Univariate analysis of Pten staining intensity and disease recurrence.

Pten expression	Relapse of disease		*P* value
	Yes	No	
Weak expression	23	74	.02
Strong expression	10	82	

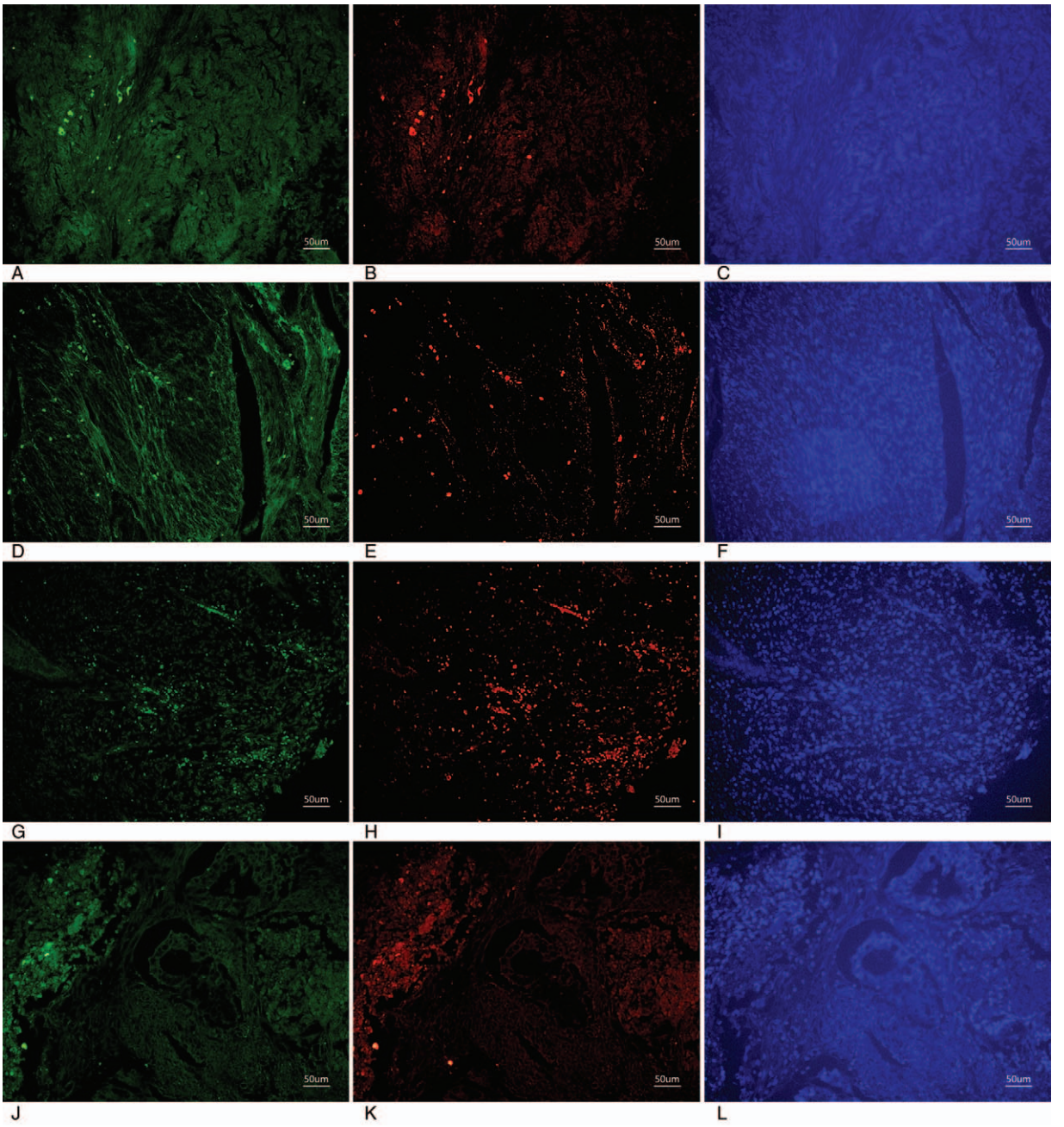

Figure 4. Immunohistochemical staining expression of $CD4^+FOXP3^+$ T cell in endometrial normal tissues and adenocarcinoma of all grades. (A-1) CD4+ T cells; (A-2) FOXP3+ T cells; (A-3) cell nuclei in highly differentiated tissue. (B-1) CD4+ T cells; (B-2) FOXP3+ T cells; (B-3) cell nuclei in moderately differentiated tissue. (C-1) CD4+ T cells; (C-2) FOXP3+ T cells; (C-3) cell nuclei in poorly differentiated tissue. (D-1) CD4+ T cells; (D-2) FOXP3+ T cells; (D-3) cell nuclei in normal tissue.

shown a negative correlation between Pten level and diabetes, with higher Pten levels in diabetes patients, which confirmed the link between diabetes and endometrial cancer. It also may suggest an intrinsic relationship between serum Pten and endometrial cancer.

5. Conclusion

In comparison with normal endometrial tissues, Pten expression in endometrial adenocarcinomas was weaker, and the degree of attenuation was roughly correlated with tumor grade. In contrast, accumulation of $CD4^+FOXP3^+$ T cells was positively correlated with tumor grade. Loss of Pten expression and accumulation of $CD4^+FOXP3^+$ T cells in lesions were inversely related to prognosis. The data suggested a possible link between serum Pten and $CD4^+FOXP3^+$ T cells in patients with endometrial cancer and may also be useful biomarkers for endometrial cancer prognosis. We also speculated that there may be a potential regulatory pathway between Pten and $CD4^+FOXP3^+$ T cells. Such pathway is worthy further investigations as a new avenue for targeted immunotherapy of endometrial adenocarcinoma.

Table 5

Relationship between CD4⁺FOXP3⁺ T infiltration and clinicopathological features in patients.

Characteristics	Number	Staining for CD4⁺FOXP3⁺ T cells: Weak (< 7.9, n=117)	Strong (≥7.9, n=83)	*P* value
Menopausal status				
Premenopausal	112	63	49	.466
Postmenopausal	88	54	34	
BMI				
< 25	118	77	41	.020
≥ 25	82	40	42	
Diabetes				
Yes	32	24	8	.039
No	168	93	75	
Hypertension				
Yes	47	33	14	.062
No	153	84	69	
FIGO stage				
I	158	95	63	.365
II/III	42	22	20	
Differentiation stage				
I	69	44	25	.003
II	77	52	25	
III	54	21	33	

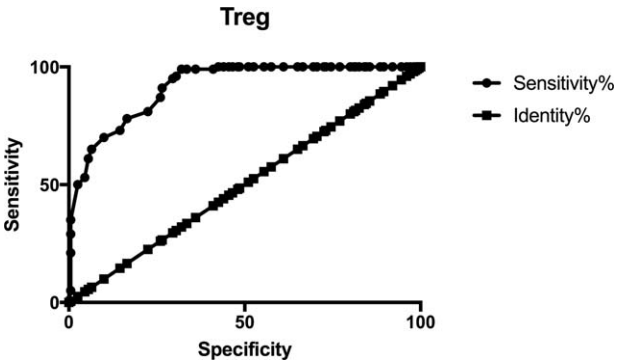

Figure 5. The ROC curves of CD4⁺FOXP3⁺ T cell expressions among the subjects.

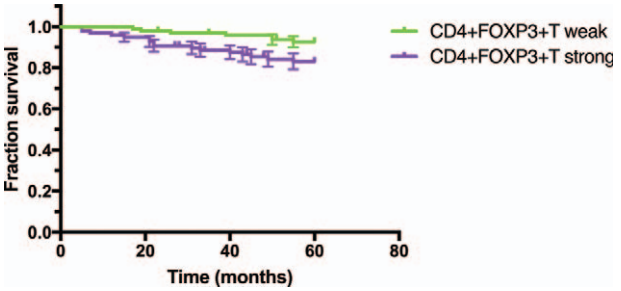

Figure 6. Relationship between prognosis and CD4⁺FOXP3⁺ T cell expression into tumors.

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Author contributions

Conceptualization: Zeng Xi, Liao Guang-Dong.
Data curation: Zeng Xi, Lan Zhu, Liao Guang-Dong.
Formal analysis: Zeng Xi, Li Jing, Liao Guang-Dong.
Funding acquisition: Li Jing, Xi Ming-Rong.
Investigation: Zeng Xi, Li Jing, Kang Le-Ni, Deng Ze-Wen, Ye Hui, Xi Ming-Rong.
Methodology: Zeng Xi, Li Jing, Kang Le-Ni, Deng Ze-Wen, Ye Hui, Xi Ming-Rong, Liao Guang-Dong.
Project administration: Zeng Xi, Lan Zhu, Ye Hui, Liao Guang-Dong.
Resources: Zeng Xi, Lan Zhu, Deng Ze-Wen, Ye Hui.
Software: Zeng Xi, Li Jing, Kang Le-Ni, Deng Ze-Wen, Ye Hui, Liao Guang-Dong.
Supervision: Zeng Xi, Lan Zhu, Xi Ming-Rong, Liao Guang-Dong.
Writing – original draft: Zeng Xi, Deng Ze-Wen, Liao Guang-Dong.
Writing – review & editing: Zeng Xi, Xi Ming-Rong, Liao Guang-Dong.

Table 6

Relationship between immunofluorescence staining for CD4⁺FOXP3⁺ T cells and clinical characteristics of control subjects.

Characters	Number	Staining for CD4⁺FOXP3⁺ T cells: Weak (<7.9, n=61)	Strong (≥7.9, n=39)	*P* value
Menopausal status				
Premenopausal	59	32	27	.096
Postmenopausal	41	29	12	
BMI				
< 25	67	38	29	.211
≥ 25	33	23	10	
Diabetes				
Yes	17	7	10	.066
No	83	54	29	
Hypertension				
Yes	12	9	3	.358*
No	88	52	36	

* By Fisher exact probability method.

Table 7

Univariate analysis of CD4⁺FOXP3⁺T cell infiltration and disease recurrence.

Staining intensity for CD4+FOXP3+ T cells	Relapse: Yes	No	*P* value
Weak expression	10	87	.013
Strong expression	22	70	

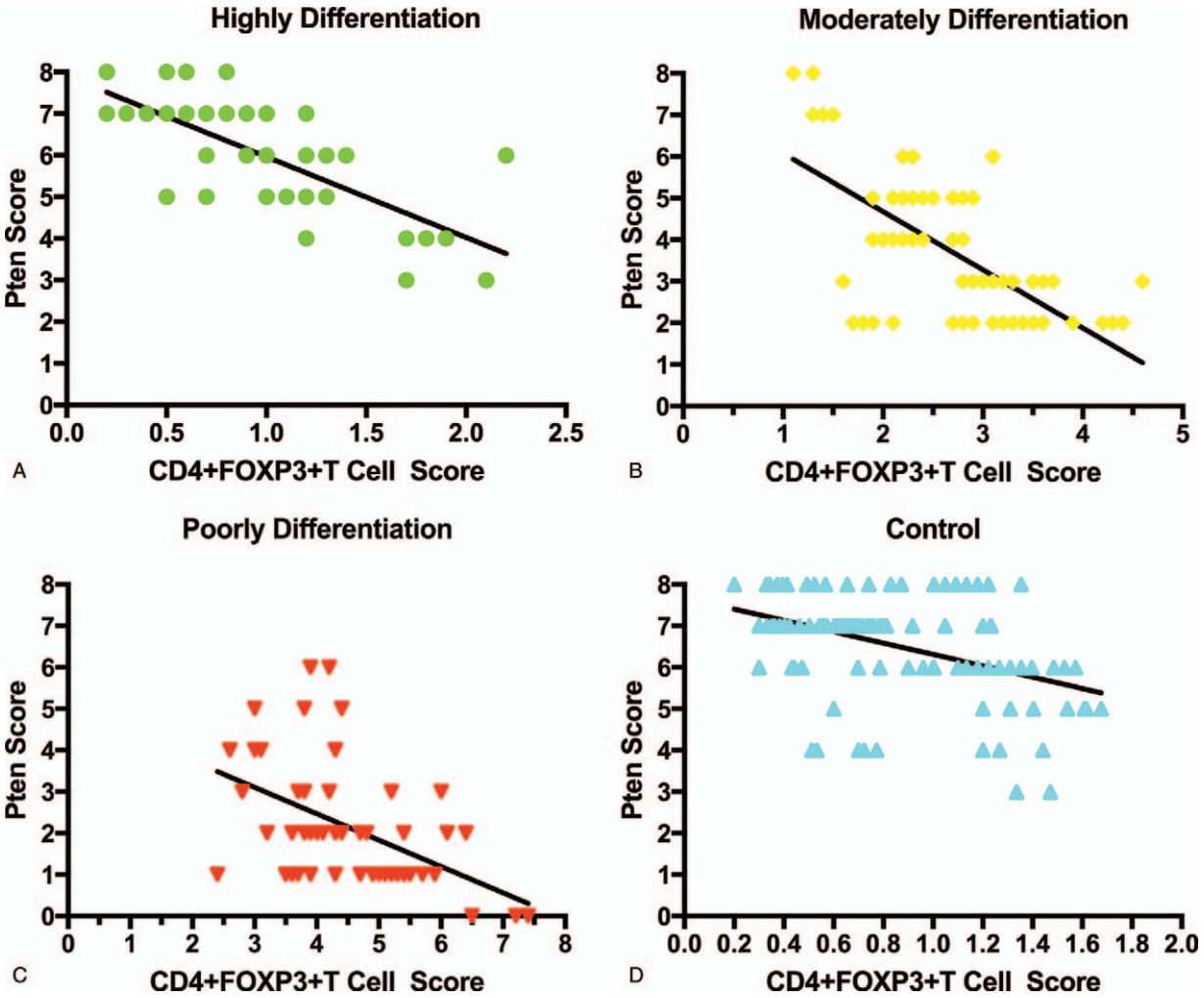

Figure 7. Pearson correlation analysis between Pten expression and $CD4^+FOXP3^+$ T cells in (A) highly differentiated tissues, $r=-0.6869$, $R^2=0.4718$, $P<.001$, 95% confidence interval (−0.7944, −0.5376); (B) moderately differentiated tissues, $r=-0.6688$, $R^2=0.4474$, $P=.032$, 95% confidence interval (−0.7765, −0.5232); (C) poorly differentiated tissues, $r=-0.4818$, $R^2=0.2341$, $P=.02$, 95% confidence interval (−0.6639, −0.2457); (D) control tissues, $r=-0.4157$, $R^2=0.1728$, $P<.001$, 95% confidence interval (−0.5659, −0.2388).

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