T cell exhaustion is associated with the risk of papillary thyroid carcinoma and can be a predictive and sensitive biomarker for diagnosis

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Research

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Abstract

Background

The incidence of papillary thyroid carcinoma (PTC) has been steadily increasing over the past decades. Hashimoto's thyroiditis (HT) is the most common autoimmune disease, and is related to the pathogenesis of PTC. Programmed cell death protein-1 (PD-1) is currently used for the treatment of PTC, but there are very few studies on the clinical value of PD-1 in the diagnosis and targeted therapy of PTC.

Methods

The expressions of PD-1 in the peripheral blood of 139 patients with PTC (PTC group), 48 patients with thyroid nodules (TN group) and 63 healthy subjects (HP group) were detected by flow cytometry. The expressions of plasma T3, T4, FT3, FT4, TSH, TGAb and TPO were detected by chemiluminescence immunoassay. T cell subsets, thyroid function indexes and PD-1 were also detected.

Results

The expressions of FT3, TGAb, $CD3^+PD\text{-}1^+$, $CD3^+CD4^+PD\text{-}1^+$ and $CD3^+CD8^+PD\text{-}1^+$ in PTC and TN were significantly higher than that in the HP group. Moreover, $CD3^+PD\text{-}1^+$, $CD3^+CD4^+PD\text{-}1^+$ and $CD3^+CD8^+PD\text{-}1^+$ expressions had significant differences between the PTC group and the TN group. In addition, the expressions of TGAb, TPO, $CD3^+PD\text{-}1^+$, $CD3^+CD4^+PD\text{-}1^+$ and $CD3^+CD8^+PD\text{-}1^+$ in PTC combined with HT (PCH group) were significantly higher than that in the PTC group. PD-1 showed a significant correlation with PTC lymph node metastasis. $CD3^+PD\text{-}1^+$ and $CD3^+CD4^+PD\text{-}1^+$ expressions were higher in N1 stage than in N0 stage.

Conclusions

The present study showed that T cell exhaustion might act as a biomarker for the differential diagnosis of PTC and TN. The combination of PD-1, TGAb and TPO might be used as an early warning biomarker for the progression of HT to PTC. Targeting the PD-1 pathway could be a new approach to treat PTC and prevent malignant transformation from HT to PCH in the future.

1. Introduction

Thyroid cancer (TC) is the most common endocrine malignancy[1], and its incidence is steadily increasing over the past decades[2]. Papillary thyroid carcinoma (PTC) is the commonest subtype of TC[3]. Recent studies have confirmed that the annual increase in the incidence of TC is mainly due to the increase in the incidence of PTC, and is related to overdiagnosis[4]. The prognosis of PTC is good, however some patients are susceptible to lymph node metastasis, which is associated with poor prognosis. Few patients are

insensitive to traditional surgery or develop postoperative recurrence. Therefore, it is important to discover new targets to improve the diagnosis and treatment of PTC. Immune cells play a vital role in the occurrence, development and prevention of tumors.

Hashimoto's thyroiditis (HT) is the most common autoimmune disease characterized by the destruction of thyroid cells by leukocytes and antibody-mediated immune processes. Compared with the normal population, HT patients have a higher probability of developing TC, but its pathogenesis remains unclear[5]. Some clinical studies have found that thyroid dysfunction may occur after use of PD-1/PD-L1 inhibitor[6].

The purpose of this study was to evaluate the level of T cell exhaustion in patients with PTC, determine whether PTC combined with HT (PCH) can further promote the decline of T cell function and proliferation, and identify new diagnostic targets for PTC and PCH. Targeting the PD-1/PD-L1 pathway may provide a new therapeutic strategy to treat PTC and prevent malignant transformation from HT to PCH in the future.

2. Material And Methods

2.1Subjects

Peripheral blood was drawn from 132 patients with PTC, 48 patients with TN and 63 normal controls who underwent surgical resection or physical examination at the Taizhou Central Hospital between June 2020 and January 2021. People with other tumors or tumor history were excluded from this study. None of the patients received chemotherapy or radiotherapy. The PTC population included 107 females and 25 males, and their age ranged from 22-80 years. There was no significant difference in age and gender among the PTC, TN and HP groups (Table 1). In addition, the patients' clinical stage, tumor stage, lymph node stage and extrathyroidal extension stage were listed according to the Tumor/Node/Metastasis (TNM) classification[7]. This study was approved by the Ethics Committee of Taizhou Central Hospital (Taizhou, PRC). Written formal consent was obtained from all subjects.

2.2Sample acquisition

Venous blood samples were collected from all subjects in the morning after overnight fasting. 3 ml of peripheral blood was collected from PTC, TN patients and HP and added to EDTA-K2 anticoagulant-containing tubes. The T, B, NK cells and PD-1 were analyzed by flow cytometry (BD FACSAria Ⅱ, USA). Then, the peripheral blood was centrifuged at 3000 rpm, and the upper layer of plasma was drawn. The plasma levels of T3, T4, FT3, FT4, TSH, TGAb and TPO (thyroid function indexes) were determined by chemiluminescence immunoassay (CLIA) with biochemical instruments (SIEMENS ADVIA Centaur XP, GER).

2.3 Flow cytometric analysis

Peripheral blood and microspheres were incubated in the dark for 30 minutes, hemolysin was added to destroy the red blood cells, then the sample was centrifuged to add PBS and analyzed immediately using BD FACSAria II cytometer and FlowJo software (BD, USA). Finally, the percentages of total T, $CD3^+CD4^+$, $CD3^+CD8^+$ T cells, NK cells, B cells, $CD4^+HLA\text{-}DR^+$, $CD8^+HLA\text{-}DR^+$, $CD8^+CD38^+$ subsets, $CD3^+PD\text{-}1^+$,

$CD3^+CD4^+PD\text{-}1^+$ and $CD3^+CD8^+PD\text{-}1^+$ were obtained. All the experiments were conducted according to the manufacturer's instructions.

2.4 Statistical analysis

All statistical analyses were conducted using SPSS v20.0 (IBM, USA) and GraphPad Prism8 (GraphPad Software, USA). If the data was normally distributed, mean ± standard deviation (SD) was used to express the data, and one-way analysis of variance and independent sample t-test was used to analyze the data. If the data was non-normally distributed, the median (Q25,Q75) was used to represent the data, and Kruskal-Wallis H test and Wilcoxon test was used to analyze the data. For data analysis between three groups, when one-way analysis of variance was used, further pairwise comparisons could be made irrespective of a significant difference. When the Kruskal-Wallis H test was used, further pairwise comparisons were made only when there was a significant difference between the three groups. A p-value < 0.05 was considered to be statistically significant.

4. Results

1. **FT3 and TGAb were markedly increased in PTC and TN patients**

We examined the T3, T4, FT3, FT4, TSH, TGAb and TPO expressions among the PTC group, TN group and HP group (Table 1, Figure 1). The results showed that the expression of FT3 in the PTC group and TN group was significantly higher than that in the HP group, while the expression of TGAb in the PTC group was significantly higher than that in the HP group ($p<0.05$). There was no significant difference in T3, T4, FT4 and TPO among the three groups ($p>0.05$).

2. **PD-1 expression in PTC, TN and HP groups**

The results revealed that the expression of $CD3^+PD\text{-}1^+$ and $CD3^+CD4^+PD\text{-}1^+$ was significantly higher in the PTC group than in the HP group, while the expression of $CD3^+$ $PD\text{-}1^+$, $CD3^+CD4^+PD\text{-}1^+$ and $CD3^+CD8^+PD\text{-}1^+$ was significantly lower in the TN group than in the HP group ($p<0.05$) (Table 1, Figure 1, Figure 4). The expression of $CD3^+PD\text{-}1^+$, $CD3^+CD4^+PD\text{-}1^+$ and $CD3^+CD8^+PD\text{-}1^+$ in the PTC group was significantly different from that in the TN group ($p<0.05$) (Table 1, Figure 1). These results suggested that PD-1 might be a diagnostic biomarker to distinguish between PTC, TN and healthy people, and can be used for thyroid nodule monitoring.

3. **The expression of immune cells, thyroid function indexes and PD-1 in PCH and PTC groups**

To determine the differences in immune cells, thyroid function indexes and PD-1 between PCH and PTC, we detected the expression of immune cells, thyroid function indexes and PD-1 among the two groups. The expression of TGAb, TPO, $CD3^+PD\text{-}1^+$, $CD3^+CD4^+PD\text{-}1^+$ and $CD3^+CD8^+PD\text{-}1^+$ was significantly higher in the PCH group than in the PTC group ($p<0.05$) (Table 2, Figure 2, Figure 4). In addition, no significant difference was observed in immune cells and thyroid function indexes among the two groups ($p>0.05$). These results indicated that PD-1, TGAb and TPO were correlated with PCH.

4. **PD-1 expression was associated with PTC lymph node metastasis**

To investigate whether PD-1, immune cells and thyroid function indexes are associated with PTC lymph node metastasis, we detected their expression levels. The expression of immune cells and thyroid function indexes showed no significant changes among the PTC N0 and PTC N1 stages (p >0.05). In contrast, the expression of $CD3^+$ $PD\text{-}1^+$ and $CD3^+CD4^+PD\text{-}1^+$ in the PTC N1 group was significantly higher than that in the N0 group (p <0.05) (Table 3, Figure 3). The above results indicated that PD-1 might be a novel prognostic indicator of PTC.

5. **The relationship between PD-1 and the diagnosis of PTC**

Using PTC clinical diagnosis as the gold standard, the ROC curves of $CD3^+PD\text{-}1^+$, $CD3^+CD4^+PD\text{-}1^+$ and $CD3^+CD8^+PD\text{-}1^+$ were drawn, with sensitivity as the ordinate and 1-specificity as the abscissa. The AUC values were compared. The AUC values were 0.770, 0,728 and 0.625, respectively (Figure 5).

4. Discussion

TC is the most common endocrine malignant tumor, with the fifth highest incidence among female tumors[2]. PTC accounts for 85% of all types of TC. Recent studies have shown that cancer cells can escape immune detection in the tumor microenvironment (TME). The normal microenvironment maintains tissue homeostasis and prevents the occurrence of tumors. Inflammatory mediators, reactive oxygen species, cytokines and chemokines from TME promote tumor growth. The relationship between TME and cancer cells is well established. The interaction between immune cells and cancer cells within TME contributes to all stages of cancer, starting from the early stage of tumorigenesis to the progression and metastasis of cancer[8, 9]. T cell immunity plays an important role in the immunosuppressive pathway. However, the immunosuppressive mechanism in TME weakens the effector function of T cells[10].

Antigen-specific T cells are the key protective factors against cancer, but continuous antigen stimulation leads to T cell exhaustion. The function and proliferation of exhausted T cells decreases, partly due to the overexpression of inhibitory receptors such as PD-1. Inflammation is associated with TC, which raises key questions about the role of immune cells in its pathogenesis. There are numerous immune cells and mediators in the TC ecosystem. Our previous study showed that $CD8^+CD38^+$T cells act as immune activation novel biomarkers and early warning indicator of patients with PTC[11]. PD-1 is highly expressed in activated T cells, B cells, dendritic cells and natural killer cells[12, 13]. Among immunosuppressive mechanisms, PD-1 has become the main marker of T cell dysfunction[14]. Many studies have shown that blocking the interaction between PD-1 and PD-L1 can reverse immune exhaustion and mediate anti-tumor activity[15-17]. PD-1 mainly restricts T cell activity in TME in the late stage of tumor growth[18]. PD-1 is activated after binding to PD-L1, and recruits phosphatase SHP-2 close to T cell receptor (TCR) and costimulatory signal to block the activation of PI3K and Akt mediated by CD28[19]. The expression of PD-L1 and the status of cytotoxic T lymphocytes (CTL) in cancer patients are considered to be the decisive factors of their overall survival rate[20]. Blocking PD-1 pathway provides a new therapeutic approach for

reinvigorating T cell response. In this study, the expressions of $CD3^+$ $PD\text{-}1^+$ and $CD3^+CD4^+$ $PD\text{-}1^+$ were significantly increased in the TN and PTC groups, and the expression of PD-1 was significantly higher in PTC patients in N1 stage than those in N0 stage.

Many recent studies have confirmed the correlation between thyroid autoimmune diseases and the incidence of PTC. HT is the most common autoimmune disease characterized by the destruction of thyroid cells by leukocytes and antibody-mediated immune processes. It causes chronic inflammation of the thyroid tissue. Many studies have demonstrated the relationship between PTC and HT. Fiore et al.[21] found that the prevalence of PTC is higher in patients with nodular-HT than in patients with nodular goiter. Moreover, 5-10% of patients with both PTC and HT may develop aggressive disease and require systemic treatment[22]. Both TPO and TgAb are independent risk factors for TC. TPO is the enzyme responsible for the production of thyroid hormones. Li et al.[23] found that positive TGAb might be a risk factor for cervical lymph node metastasis. In this study, the expression of TPO and TGAb was significantly higher in the PCH group than in the PTC group. However, there was no significant difference between thyroid function indexes and PTC lymph node metastasis. Meanwhile, we found that the expression of $CD3^+PD\text{-}1^+$, $CD3^+CD4^+PD\text{-}1^+$ and $CD3^+CD8^+PD\text{-}1^+$ was significantly higher in PCH patients than in PTC without HT patients, suggesting that PD-1 might act as an early warning indicator of the progression of HT patients to PTC.

In this study, we measured the expression of $CD3^+CD4^+$, $CD3^+CD8^+$ T cells, NK cells, B cells, $CD4^+HLA\text{-}DR^+$, $CD8^+HLA\text{-}DR^+$, $CD8^+CD38^+$, $CD3^+PD\text{-}1^+$, $CD3^+CD4^+PD\text{-}1^+$ and $CD3^+CD8^+PD\text{-}1^+$ by flow cytometry, and T3, T4, FT3, FT4, TSH, TGAb and TPO by chemiluminescence immunoassay (CLIA) among PTC patients (PTC group), thyroid nodule patients (TN group) and normal population (HP group) to determine whether they were related to the progression of PTC. We also explored the differences in $CD3^+CD4^+$, $CD3^+CD8^+$ T cells, NK cells, B cells, $CD4^+HLA\text{-}DR^+$, $CD8^+HLA\text{-}DR^+$, $CD8^+CD38^+$ subsets, $CD3^+PD\text{-}1^+$, $CD3^+CD4^+$ $PD\text{-}1^+$ and $CD3^+CD8^+PD\text{-}1^+$, T3, T4, FT3, FT4, TSH, TGAb and TPO expression between the PCH group and the PTC group.

In summary, $PD\text{-}1^+$T cells might act as a biomarker for the differential diagnosis of PTC and TN. The combination of PD-1, TGAb and TPO might be used as an early warning biomarker for the progression of HT to PTC. Targeting the PD-1 pathway could be a new approach to treat PTC and prevent malignant transformation from HT to PCH in the future.

Declarations

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

No conflict of interest exits in the submission of this manuscript, and manuscript is approved by all authors for publication.

Ethics statement

This study was approved by Ethics Committee of Taizhou Central Hospital. Written informed consent was obtained from each individual in the study.

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Tables

Table 1 Papillary thyroid carcinoma patients’ clinicopathological characteristics and laboratory data

	PTC	TN	HP	*P*	*P1*	*P2*	*P3*
n	132	48	63				
Age [year]	49[22~80]	52[29~71]	47[26~74]	0.06			
Gender				0.16			
Male	25	6	17				
Female	107	42	46				
T							
T1+T2	130						
T3+T4	2						
N							
N0	83						
N1a+N1b	49						
M							
M0	131						
M1	1						
Clinical stage							
I	122						
II	10						
Laboratory data							
T3[nmol/L]	1.18 [1.06,1.28]	1.23[1.12,1.31]	1.21 [1.12,1.31]	0.23			
T4[nmol/L]	8.20 [7.20,9.10]	8.40 [7.15,9.00]	8.00 [7.00,8.50]	0.22			
FT3[pmol/L]	3.36 ± 0.41	3.39 ± 0.35	3.22 ± 0.36	0.04*	0.03*	0.03*	0.61
FT4[pmol/L]	1.32 ± 0.19	1.34 ± 0.17	1.32 ± 0.15	0.69	0.92	0.44	0.43
TSH[μIU/mL]	1.55 [1.07,2.09]	1.64[0.99,2.80]	1.81[1.15,2.56]	0.24			
TGAb[IU/mL]	23.5[15.0,74.5]	21.0 [15.0,29.8]	16.0[15.0,28.0]	0.00*	0.00*	0.36	0.36
TPO[IU/mL]	34.0[28.0,61.0]	28.0[28.0,38.7]	38.0[28.0,55.0]	0.08			
$CD3^+$ $PD\text{-}1^+$ (%)	11.5 ± 4.7	5.9 ± 2.1	8.6 ± 4.5	0.00*	0.00*	0.00*	0.00*

$CD3^+CD4^+PD\text{-}1^+$ (%)	7.9 ± 3.2	4.3 ± 1.8	6.43± 2.7	0.00*	0.00*	0.00*	0.00*
$CD3^+CD8^+PD\text{-}1^+$ (%)	5.1 [2.3,6.0]	2.8 [1.7,3.3]	4.7[2.7,5.8]	0.00*	0.75	0.00*	0.00*

Data were expressed as mean ± standard deviation (SD), median [interquartile range].The continuous variables were compared by using the Student's t-test and the Mann-Whitney U test, the categorical variables were compared by using the χ2 or Fisher's exact test between discovery and validation groups. *P*:statistical difference within each group, *P1*: PTC and HP, *P2*: TN and HP, *P3*: PTC and TN

Table 2 The changes of lymphocyte subsets, thyroid function indexes and PD-1 among PCH and PTC

	PCH(n=49)	PTC (n=83)	*P*
Total T(%)	62.38 ± 7.63	63.49 ± 8.49	0.454
$CD3^+CD4^+$(%)	36.39 ± 5.93	37.24 ± 6.79	0.468
$CD3^+CD8^+$(%)	22.56 ± 5.46	22.99 ± 7.40	0.705
$CD4^+/CD8^+$(%)	1.69 (1.25,2.18)	1.64 (1.19,2.37)	0.923
$CD4^+$ $HLA\text{-}DR^+$(%)	6.34 ± 2.10	5.96 ± 2.06	0.302
$CD8^+$ $HLA\text{-}DR^+$(%)	6.60 ± 2.84	6.6.5 ± 3.88	0.929
$CD8^+CD38^+$(%)	5.70 ± 2.82	5.42 ± 2.81	0.582
NK(%)	18.23 ± 8.47	17.24 ± 8.20	0.509
B(%)	15.33 ± 4.71	14.58 ± 5.10	0.398
T3(nmol/L)	1.18 ± 0.25	1.20 ± 0.20	0.660
T4(nmol/L)	8.20 (6.00,9.25)	8.20 (6.44,9.10)	0.923
FT3(pmol/L)	3.31 (3.00,3.48)	3.40 (2.92,3.57)	0.191
FT4(pmol/L)	1.35 ± 0.20	1.30 ± 0.18	0.151
TSH(μIU/mL)	1.87 ± 1.09	1.58 ± 0.85	0.086
TGAb(IU/mL)	125.00 (33.00,243.50)	18.00 (15.00,23.00)	0.000*
TPO(IU/mL)	258.00 (36.00,909.00)	28.00 (28.00,37.50)	0.000*
$CD3^+$ $PD\text{-}1^+$ (%)	13.31 ± 5.04	10.35 ± 4.13	0.000*
$CD3^+CD4^+PD\text{-}1^+$ (%)	9.24 ± 3.20	7.12 ± 2.93	0.000*
$CD3^+CD8^+PD\text{-}1^+$ (%)	5.51 ± 2.22	4.53 ± 2.13	0.013*

Table 3 Correlation between lymphocyte subsets, thyroid function index and lymph node metastasis of PTC patients

	N0 (n=83)	N1 (n=49)	*P*
Total T(%)	63.17 ± 7.26	62.92 ± 9.59	0.877
$CD3^+CD4^+$(%)	36.58 ± 6.41	37.50 ± 6.60	0.436
$CD3^+CD8^+$(%)	23.28 ± 6.27	22.07 ± 7.44	0.321
$CD4^+/CD8^+$(%)	1.64 (1.24,2.12)	1.76 (1.23,2.32)	0.382
$CD4^+$ HLA-DR^+(%)	6.06 ± 2.05	6.16 ± 2.14	0.802
$CD8^+$ HLA-DR^+(%)	6.70 (4.60,9.05)	6.00 (3.60,7.30)	0.059
$CD8^+CD38^+$(%)	4.80 (3.40,6.45)	5.40 (3.50,8.60)	0.084
NK(%)	17.81 ± 7.46	17.26 ± 9.59	0.715
B(%)	14.76 ± 4.75	15.01 ± 5.33	0.776
T3(nmol/L)	1.18 ± 0.22	1.20 ± 0.23	0.596
T4(nmol/L)	8.30 (7.15,9.15)	8.00 (7.30,8.80)	0.606
FT3(pmol/L)	3.38 (3.14,3.52)	3.37 (3.20,3.57)	0.655
FT4(pmol/L)	1.33 ± 0.20	1.31 ± 0.17	0.670
TSH(μIU/mL)	1.65 ± 0.92	1.71 ± 1.01	0.766
TGAb(IU/mL)	25.00 (15.50,78.00)	22.00 (15.00,52.00)	0.363
TPO(IU/mL)	37.00 (28.00,74.00)	30.00 (28.00,52.00)	0.188
$CD3^+$ PD-1^+ (%)	10.76 ± 4.68	12.63 ± 4.52	0.026*
$CD3^+CD4^+$PD-1^+ (%)	7.27 ± 3.01	8.98 ± 3.24	0.003*
$CD3^+CD8^+$PD-1^+ (%)	4.66 ± 1.81	5.29 ± 2.73	0.156

Figures

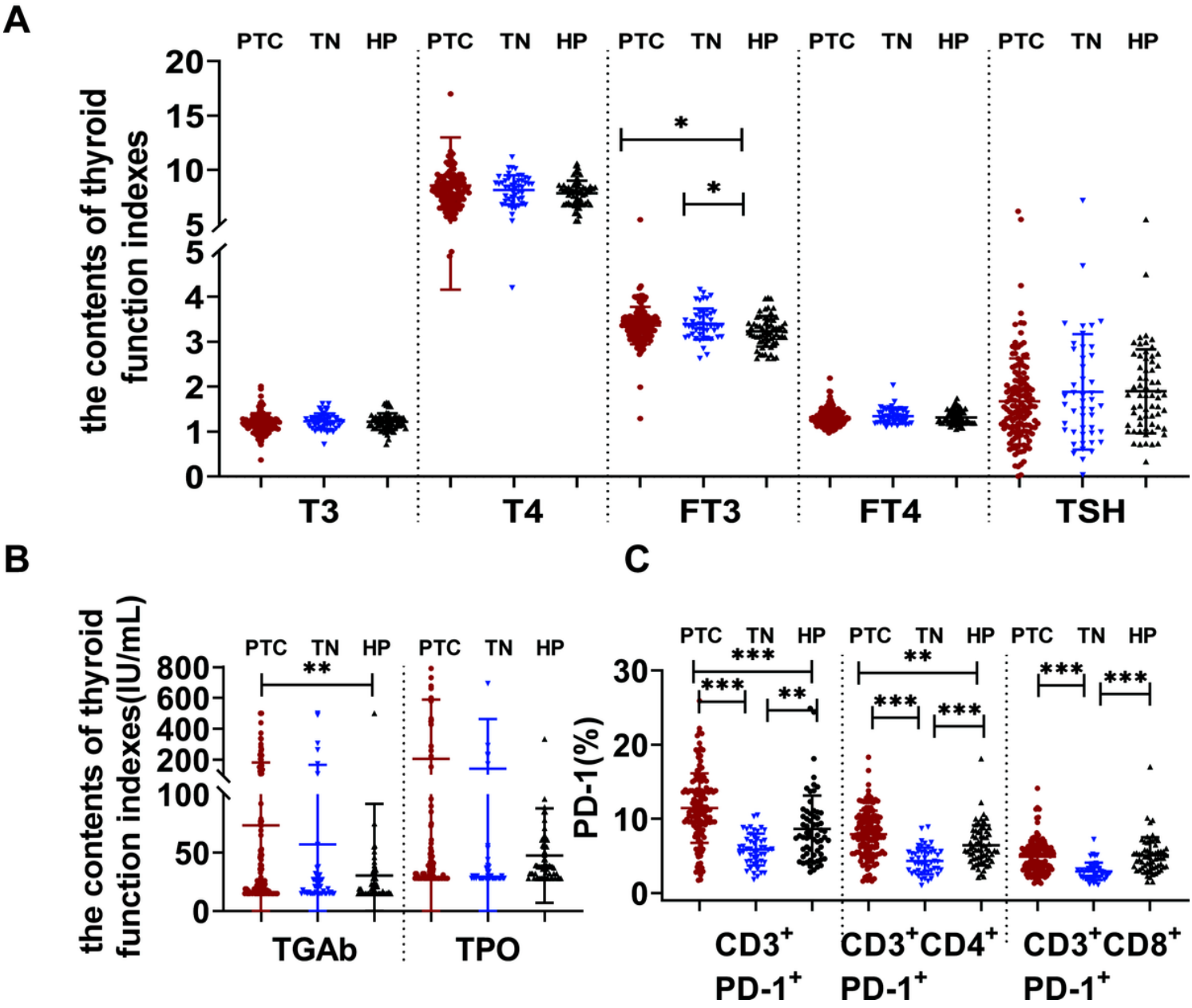

Figure 1

The activities of thyroid function indexes and PD-1. A. The plasma activities of the T3, T4, FT3, FT4, TSH among PTC, TN and HP groups. B. The plasma activities of the TGAb and TPO among PTC, TN and HP groups. C. The activities of the CD3+PD-1+, CD3+CD4+PD-1+ and CD3+CD8+PD-1+ among PTC, TN and HP groups(*P<0.05), P<0.05 represents a significant difference.

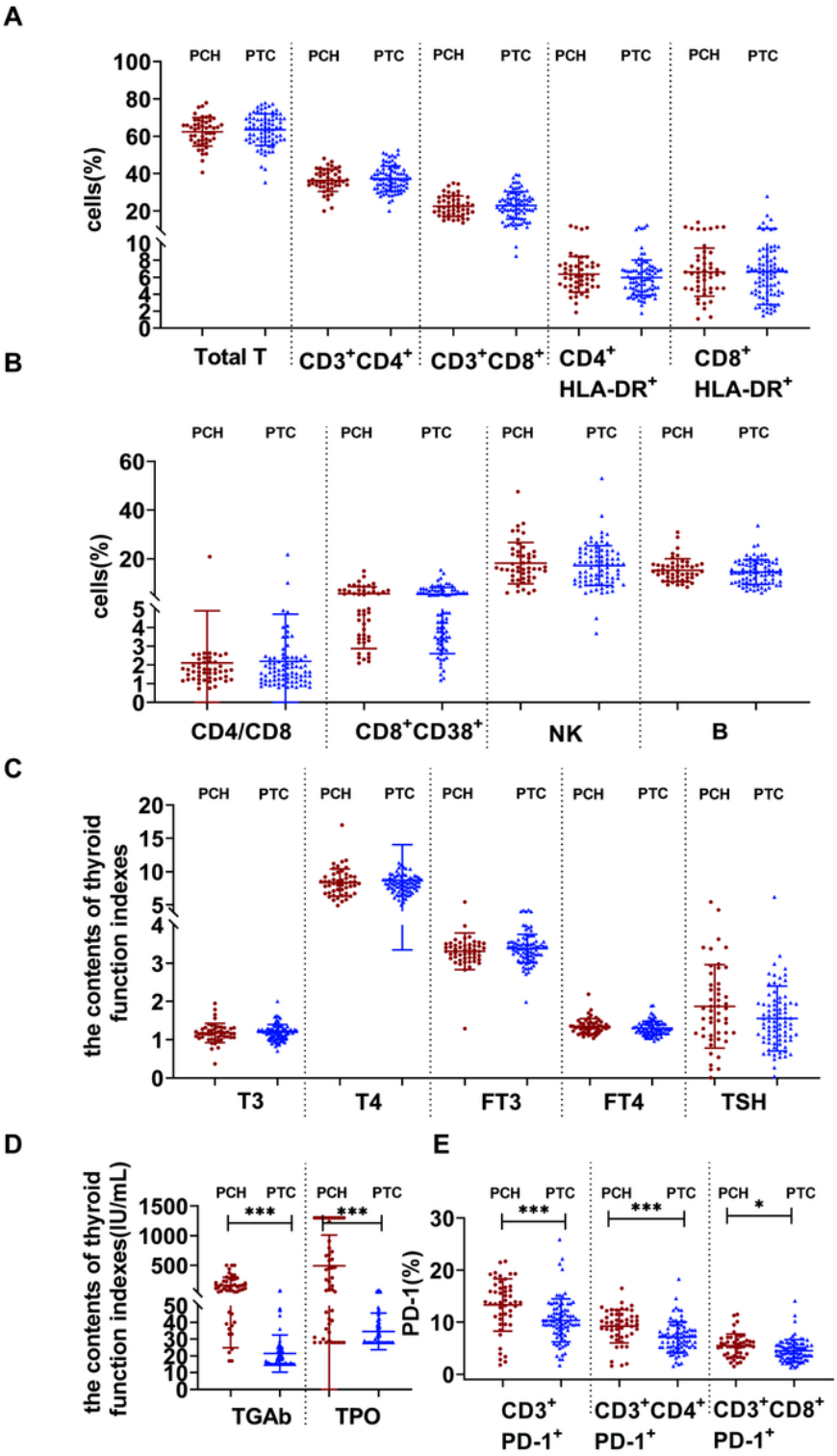

Figure 2

The activities of the NK cell, B cell, T cell subset, thyroid function indexes and PD-1. A. The activities of the Total T cells, CD3+CD4+, CD3+CD8+, CD4+HLA-DR+ and CD8+HLA-DR+ cells among PCH and PTC. B. The activities of the CD4+/CD8+, CD8+CD38+ cells,NK and B cells among PCH and PTC. C. The plasma activities of the T3, T4, FT3, FT4, TSH among PCH and PTC. D. The plasma activities of the TGAb and TPO among PCH and PTC. E. The activities of the CD3+PD-1+, CD3+CD4+PD-1+ and CD3+CD8+PD-1+ among PCH and PTC(*P<0.05), P<0.05 represents a significant difference..

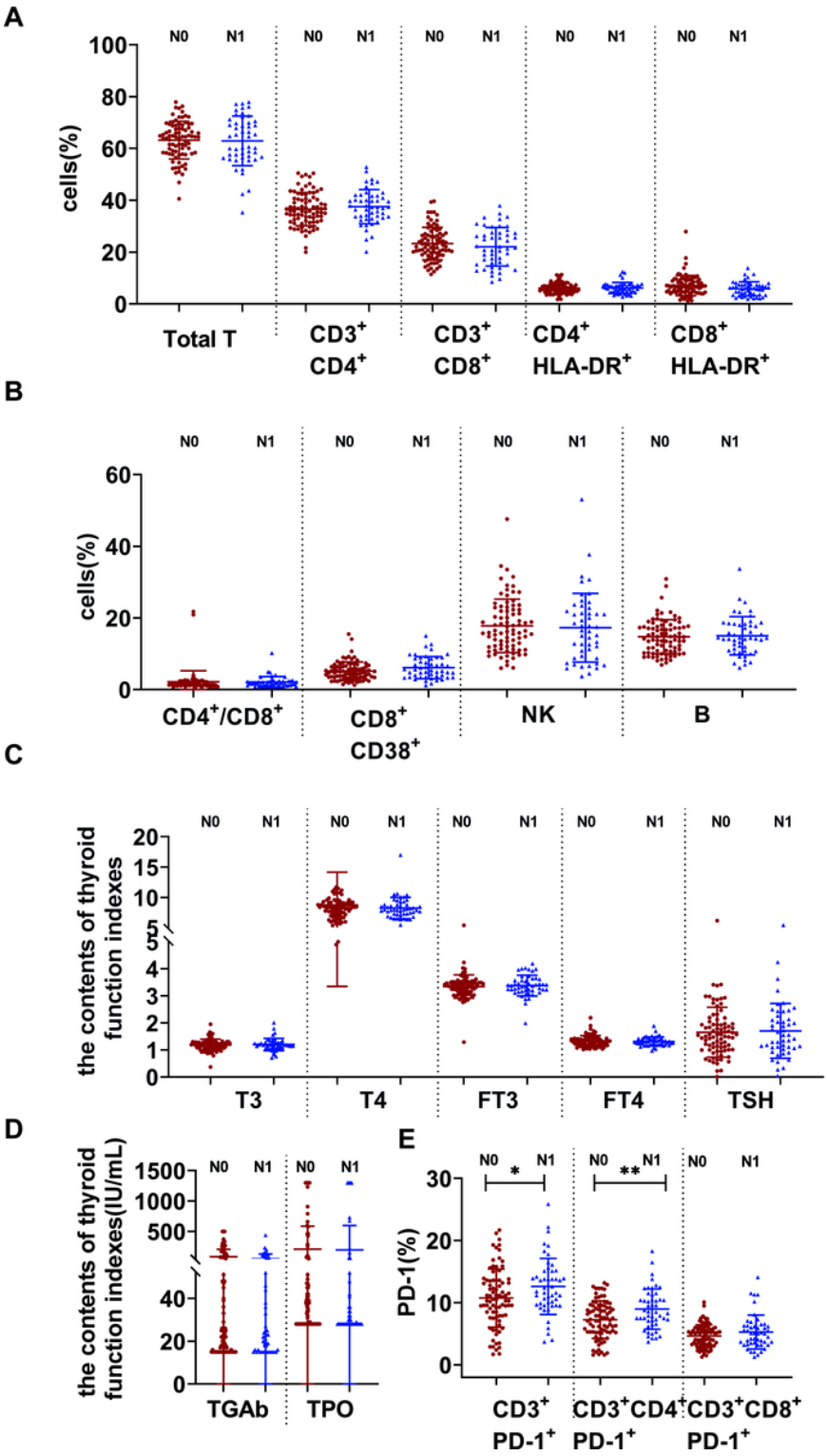

Figure 3

The activities of the NK cell, B cell, T cell subset, thyroid function indexes and PD-1. A. The activities of the Total T cells, CD3+CD4+, CD3+CD8+, CD4+HLA-DR+ and CD8+HLA-DR+ cells among N0 and N1. B. The activities of the CD4+/CD8+, CD8+CD38+ cells,NK and B cells among N0 and N1. C. The plasma activities of the T3, T4, FT3, FT4, TSH among N0 and N1. D. The plasma activities of the TGAb and TPO among N0 and N1. E. The activities of the CD3+PD-1+, CD3+CD4+PD-1+ and CD3+CD8+PD-1+ among N0 and N1(*P<0.05), P<0.05 represents a significant difference.

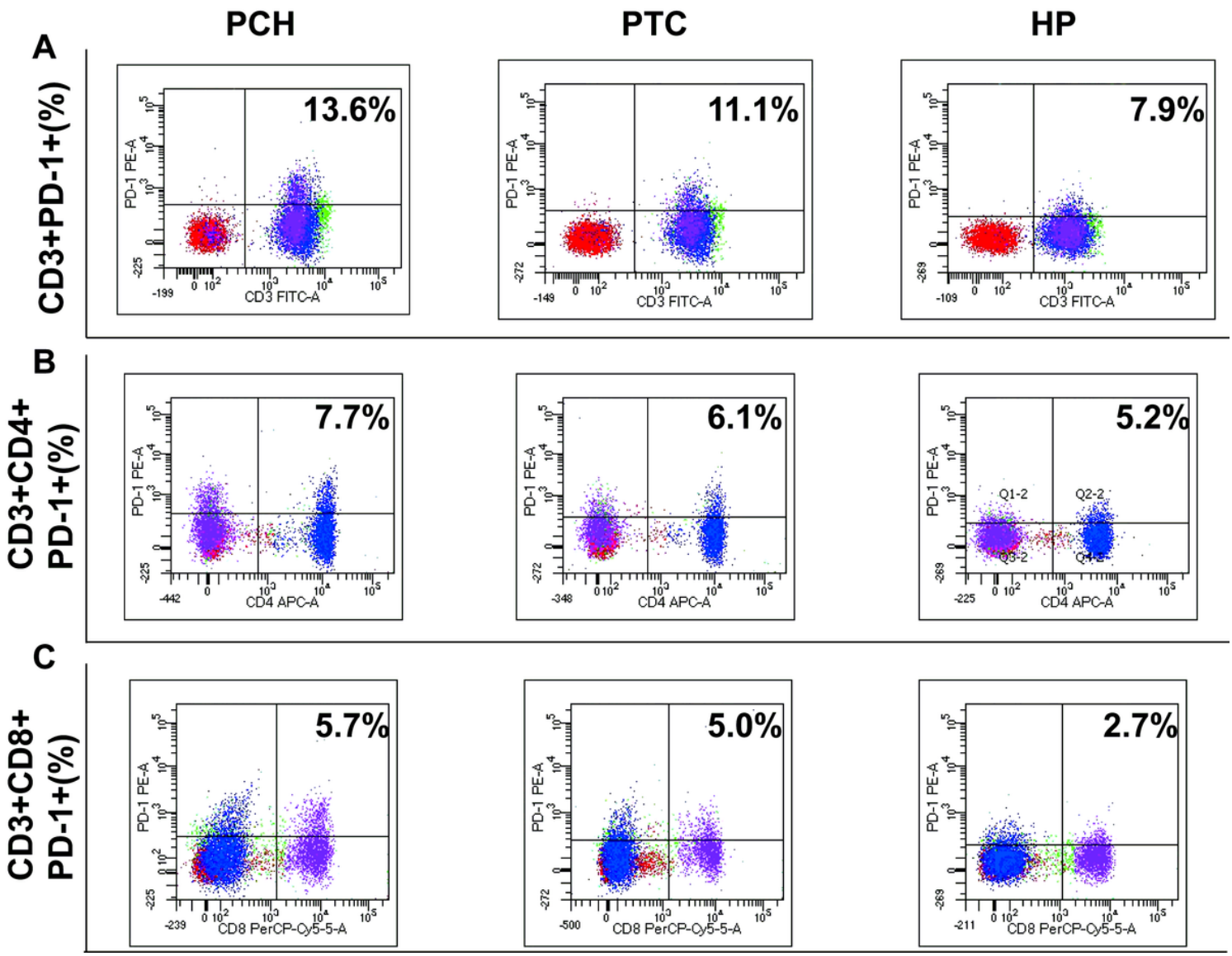

Figure 4

The expression of PD-1 in peripheral blood of PCH, PTC and HP. A. The CD3+PD-1+ expression among PCH, PTC and HP. B. The CD3+CD4+PD-1+ expression among PCH, PTC and HP. C. The CD3+CD8+PD-1+ expression among PCH, PTC and HP.

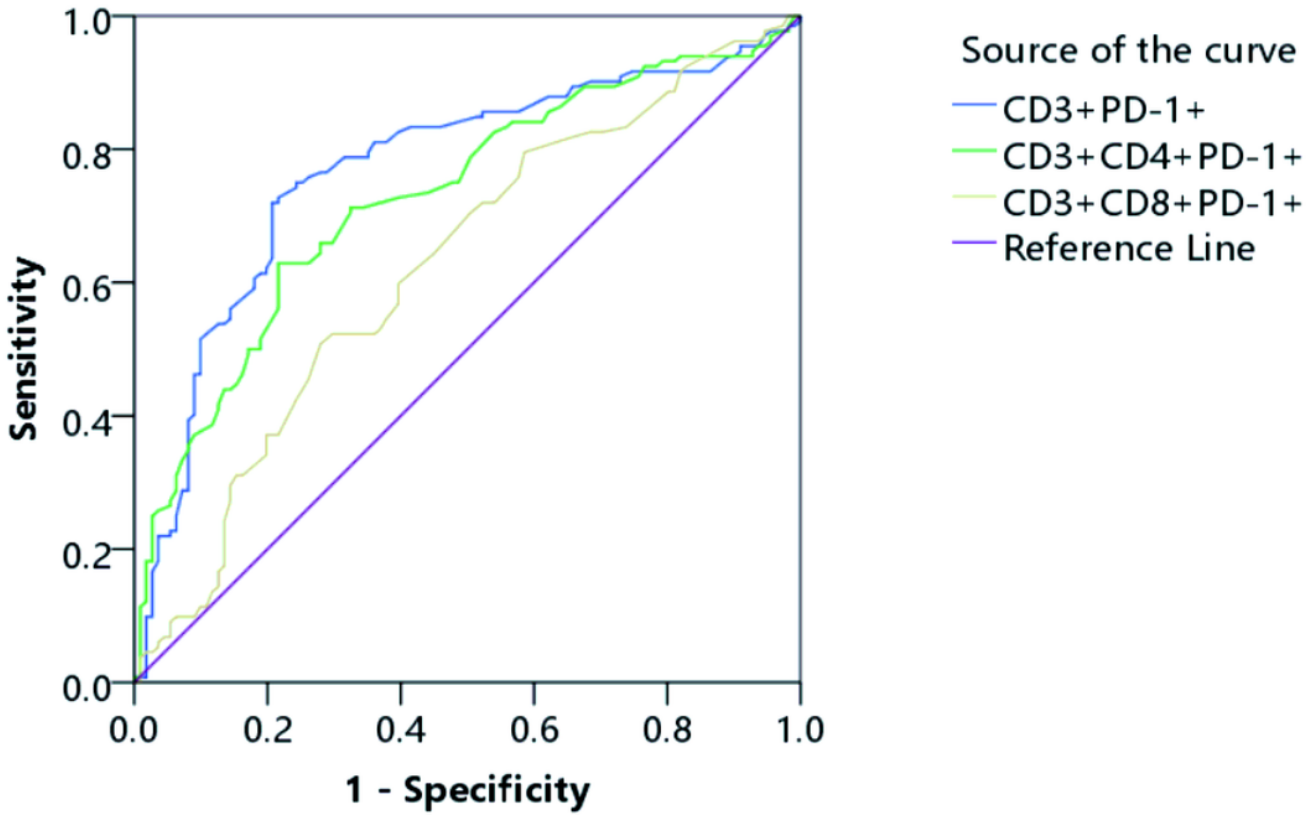

Figure 5

The ROC curve of CD3+PD-1+, CD3+CD4+PD-1+ and CD3+CD8+PD-1+.