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Review Article

Predictive and prognostic significance of T cell surface CD28 and CTLA-4 levels in hemophilia

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Abstract: Objective: To investigate the predictive and prognostic significance of expression levels of peripheral blood T cell surface CD28 and cytotoxic T lymphocyte-associated protein 4 (CTLA-4) in hemophilia. Methods: This study included 114 hemophilia patients (research group (RG)) and 120 healthy controls (control group (CG)) who were admitted to our hospital from the same period. The T cell surface CD28 and CTLA-4 levels in peripheral blood were detected and compared, and their correlation with bleeding degree and clinical effects was investigated. Results: Compared with the CG, CD28 was notably lower while CTLA-4 was dramatically higher in the RG before treatment ($P<0.05$). After treatment, CD28 increased markedly and CTLA-4 reduced dramatically in the RG compared with the CG ($P<0.05$). The degree of bleeding was significantly and negatively correlated with CD28 ($r=-0.3150$, $P<0.05$), while it was positively correlated with CTLA-4 ($r=0.4443$, $P<0.05$). As to their correlations with clinical effect, CD28 was positively correlated with clinical effect ($r=0.6838$, $P<0.05$), while CTLA-4 was negatively correlated ($r=-0.7081$, $P<0.05$). The effective group presented significantly higher CD28 level and statistically lower CTLA-4 level than the ineffective group (both $P<0.05$). Conclusion: CD28 is significantly decreased on the surface of peripheral blood T cells in hemophilia patients, while CTLA-4 is markedly increased. CD28 and CTLA-4 expression levels can be used for early clinical diagnosis and prediction of disease progression in hemophilia patients.

Keywords: CD28, CTLA-4, hemophilia, bleeding degree

Introduction

Hemophilia is a type of hereditary coagulopathy [1], of which hemophilia A is a rare factor VIII gene mutation that causes hereditary x-linked hemorrhagic disease that accounts for 85% of all cases [2]. Hemophilia is one of the most common single-gene diseases affecting the British population [3], while Brazil is the fourth largest country with hemophilia worldwide [4]. The treatment of hemophilia varies from country to country and from region to region. The quality of life score of hemophilia patients in China is reported to be much lower than that of patients in Europe [5]. In severe cases, patients need a lifetime of alternative therapy and prevention to prevent fatal hemorrhage [6]. Studies have shown that in hemophilia, repeated joint bleeding can lead to hemophilia arthropathy due to biochemical changes in joint structure caused by blood, resulting in chronic joint damage, in which the ankle and elbow joints deteriorate most frequently [7, 8].

According to statistics, one third of patients with severe hemophilia A developed antibodies against factor VIII after protein replacement therapy. While regulatory T cells (Tregs) have a natural inhibitory function, which can reduce the immune response to factor VIII treatment, and antigen-specific Tregs can effectively inhibit the anti-factor VIII antibody response [9]. CD28 provides costimulatory signals needed for T cell activation [10], and the CTLA-4 checkpoint regulates T cell activation [11]. CD28, by facilitating the development of Tregs and proliferation in the aspect of maintaining the pool size of Tregs, is crucial in maintaining the size of the regulatory T cell pool, and exerts different effects on the homeostasis of each regulatory T cell subpopulation [12]. While CTLA-4 is a key negative regulator of T-cell immunity,

which can inhibit the anti-tumor immune response [13], and its deficiency will lead to severe lymphoproliferative diseases [14].

In this paper, we measured the expression levels of CD28 and CTLA-4 in two groups of patients, analyzed their correlations with the bleeding degree and clinical efficacy, and detected the specificity and sensitivity of CD28 and CTLA-4 on the surface of peripheral blood T cells individually and jointly, aiming to explore the predictive and prognostic significance of CD28 and CTLA-4 expression levels in hemophilia.

Materials and methods

General information

One hundred and fourteen hemophilia patients, with an average age of (15.43±2.14) and an average body mass index (BMI) of (22.63±2.05), who were treated in the Third People's Hospital of Qingdao were selected as the RG. Regarding the degree of bleeding, the number of cases of mild, moderate and severe bleeding were 38, 49 and 27 respectively. In addition, another 120 healthy controls during the same period were assigned in the CG, with an average age of (14.96±2.34) and a mean BMI of (22.57±2.16).

Inclusion criteria: All the participants were accompanied by their family members at admission, with complete clinicopathological data, and patients in the RG were diagnosed wtih hemophilia. Exclusion criteria: Patients with previous history or family history of mental illness, autoimmune system defect, severe organ disease history, drug dependence history, or those who could not cooperate with examination due to aphasia, irritability, unconsciousness or communication impairment were excluded.

This study was approved by the Medical Ethics Committee of the Third People's Hospital of Qingdao, and was conducted in accordance with the Declaration of Helsinki. The contents of the experiment were described in detail to the participants and their families in advance, and all enrolled patients and healthy controls provided written informed consent to participate.

Methods

Treatment methods of patients in the RG: During the hospital stay, patients in the RG were infused weekly with coagulation factor VIII (50 IU/kg; Saihongrui Biotechnology Co., Ltd., Nanjing, China, PRO-331). The dose was adjusted based on the basis of bleeding, and was set to the standard preventive dose if the bleeding continued. In addition, the patients' bad eating habits were adjusted and other symptomatic support treatments were given, such as adjusting the patients' rest and diet, maintaining moderate rehabilitation exercise and keeping adequate sleep.

Detection of CD28 and CTLA-4 expression levels: Fasting peripheral venous blood (3-4 mL) was drawn from hemophilia patients upon admission and after treatment, and from healthy controls in the early morning of physical examination, and then the blood was placed in anticoagulant tubes. Detection of CD28 and CTLA-4 levels on the surface of peripheral blood T cells: Peripheral blood mononuclear cells (PBMCs) were isolated from 3 mL peripheral venous blood with separating medium (Yuanmu biotechnology Co., Ltd., Shanghai China, YS6132) and washed with PBS (LMAI Biological Engineering Co., Ltd., Shanghai, China, LM0221A) for 3 times before they were adjusted to a concentration of 1×10^6/L. The above samples, with a total of 100 μl each, were put into four test tubes with 20 μL of: FITC-CD4, PE-CTLA-4, PC-CD3; FITC-CD4, PE-CD28, PC-CD3; FITC-CD8, PE-CTLA-4, PC-CD3; FITC-CD8, PE-CD28 and PC-CD3, respectively. After 30 min of dark incubation at room temperature, they were detected by flow cytometry (Imagetd Trading Co., Ltd., Beijing, China, AMG0002051). The cells were obtained and analyzed by Cell Quest software, and the ratio of CD28 and CTLA-4 positive cells was determined to represent the expression percentage of each molecule.

Outcome measures

The comparison of CD28 and CTLA-4 expression levels in the two groups as well as that within the research group before and after treatment were performed. The correlation of CD28 and CTLA-4 with the bleeding degree and clinical effect was explored. CD28 and CTLA-4 levels were detected singly and jointly,

Table 1. Comparison of general data between the two groups (x±s)/[n (%)]

	RG (n=114)	CG (n=120)	t/X^2	P
Average age (years old)	15.43±2.14	14.96±2.34	1.60	0.11
Body mass index (kg/m^2)	22.63±2.05	22.57±2.16	0.22	0.83
Diastolic blood pressure (mmHg)	78.43±10.26	77.98±11.04	0.32	0.74
Systolic blood pressure (mmHg)	115.21±13.47	114.78±14.11	0.24	0.81
Average height (cm)	170.24±10.51	171.34±10.67	0.79	0.43
Respiratory rate (times/min)	16.21±2.04	15.87±2.12	1.25	0.21
Heart rate (times/min)	72.51±4.89	71.97±4.76	0.86	0.39
Ethnicity			0.90	0.34
Han (cases)	98 (85.96)	108 (90.00)		
Ethnic minorities (cases)	16 (14.04)	12 (10.00)		

and the specificity, sensitivity and area under the ROC curve (area under curve (AUC)) were analyzed.

Statistical methods

All statistical analyses of the experimental results were performed using SPSS 20.0 (IBM Corp, Armonk, NY, USA), and all graphical results were plotted by GraphPad Prism 7 (GraphPad Software Inc., San Diego, USA). The counting data are represented by [n (%)], and inter-group comparisons were conducted by the chi-square test. The measured data are described in the form of (x±s), and inter-group comparisons were carried out by the t test. The diagnostic and predictive value was analyzed by receiver operating characteristic (ROC) curve, and the correlation was analyzed by Spearman correlation coefficient. P<0.05 indicates a statistically significant difference.

Results

Comparison of general information

Patients' general clinical data, including age, body mass index (BMI), diastolic blood pressure (DBP) and systolic blood pressure (SBP), average height, respiratory rate, heart rate (HR) and ethnicity did not differ significantly between the two groups (P>0.05) (**Table 1**).

Comparison of CD28 and CTLA-4 levels between the RG and the CG before treatment

Comparison of pre-treatment CD28 and CTLA-4 levels between the RG and CG is shown in **Figure 1**. Before treatment, the CD28 level in the RG (4.37±1.12%) was notably lower than that in the CG (6.14±1.49%) (t=10.23, P<0.05), and the CTLA-4 in the RG (10.81±2.35%) was markedly higher than that in the CG (7.89±1.62%) (t=11.11, P<0.05).

Comparison of CD28 and CTLA-4 levels before and after treatment in the RG

The pre- and post-treatment CD28 and CTLA-4 levels in the RG were compared, as shown in **Figure 2**. After treatment, CD28 level (6.78±1.56%) elevated dramatically in the RG (t=13.40, P<0.05), while CTLA-4 level (8.84±1.71%) reduced noticeably (t=7.24, P<0.05).

Correlation of CD28 and CTLA-4 levels with bleeding degree in patients with hemophilia in the RG

The correlation between the expression levels of CD28 and CTLA-4 and the bleeding degree in the RG is shown in **Figure 3**. There was a significant negative correlation between CTLA-4 and bleeding degree (r=-0.3150, P<0.05), and a marked positive correlation between CD28 and bleeding degree in hemophilia patients (r=0.4443, P<0.05).

Correlation of CD28 and CTLA-4 levels with clinical effects in hemophilia patients in the RG

In the RG, 35 cases were significantly effective, 62 cases were improved and 17 cases were ineffective after treatment. The correlation between the expression levels of CD28 and CTLA-4 and the clinical efficacy of hemophilia was analyzed, as shown in **Figure 4**. It showed that CD28 was proportional to clinical effects (r=0.6838, P<0.05), and CTLA-4 was negatively related to clinical effects (r=-0.7081, P<0.05). Further, patients were classified into an effective group (n=97) and an ineffective group (n=17) based on treatment efficacy. CD28 level in the ineffective group was found to be greatly lower, and the CTLA-4 level was obviously high-

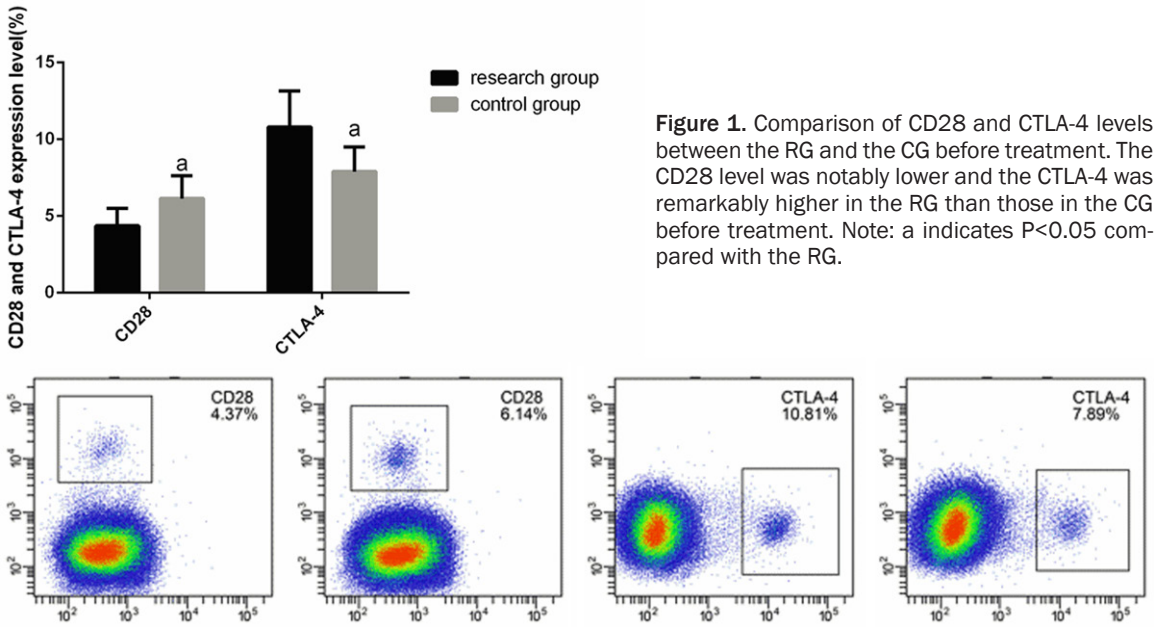

Figure 1. Comparison of CD28 and CTLA-4 levels between the RG and the CG before treatment. The CD28 level was notably lower and the CTLA-4 was remarkably higher in the RG than those in the CG before treatment. Note: a indicates P<0.05 compared with the RG.

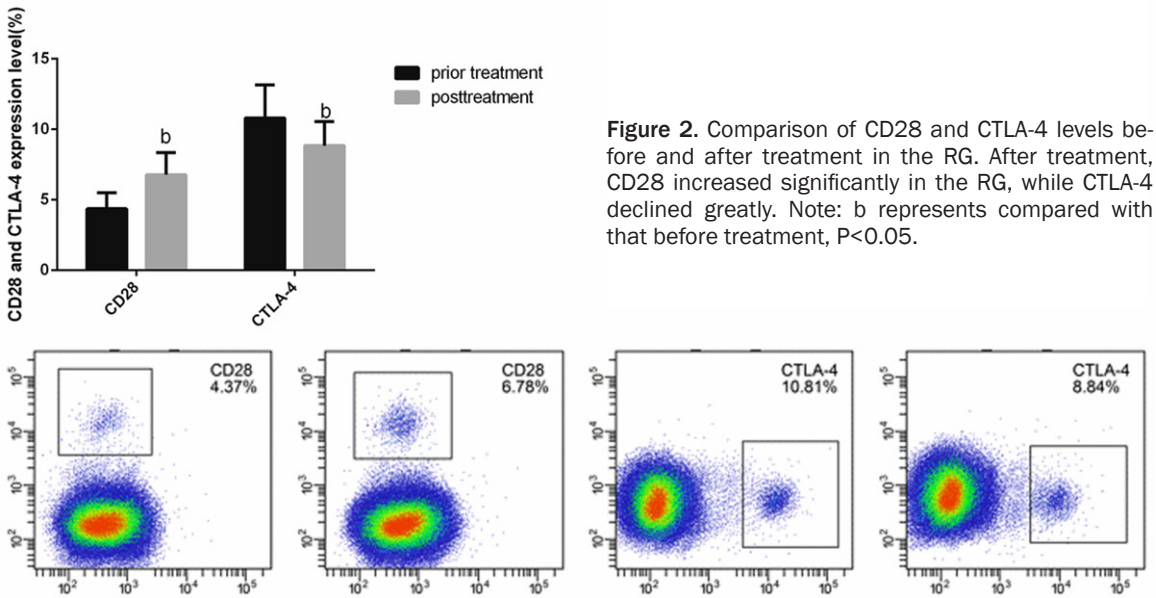

Figure 2. Comparison of CD28 and CTLA-4 levels before and after treatment in the RG. After treatment, CD28 increased significantly in the RG, while CTLA-4 declined greatly. Note: b represents compared with that before treatment, P<0.05.

er compared with the effective group (P<0.05). (**Table 2**).

Value of single and combined detection of CD28 and CTLA-4 in predicting the diagnosis of hemophilia in the two groups before treatment

Single and joint detection of CD28 and CTLA-4 in the two groups before treatment showed that CTLA-4 had the highest sensitivity (85.83%), and the combined detection had the highest specificity (89.47%) and the highest AUC (0.91), as shown in **Table 3** and **Figure 5**.

Value of single and combined detection of CD28 and CTLA-4 in predicting the clinical effects of hemophilia before treatment in the effective and ineffective groups

The single and combined detection of CD28 and CTLA-4 levels in the effective and ineffective groups before treatment showed that CD28 enjoyed the highest specificity (85.94%),

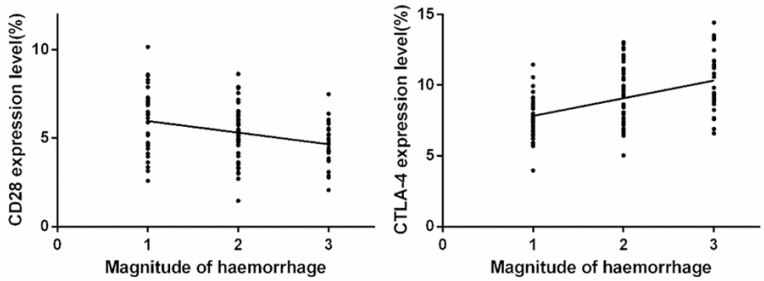

Figure 3. Correlation of CD28 and CTLA-4 levels with hemophilia bleeding degree in the RG. CD28 was negatively associated with the bleeding degree, and its level decreased with the increase of bleeding degree in patients. CTLA-4 was in positive correlation with bleeding degree, and its level increased as the degree of bleeding increased. Note: 1 represents slight bleeding, 2 represents moderate bleeding, and 3 represents severe bleeding.

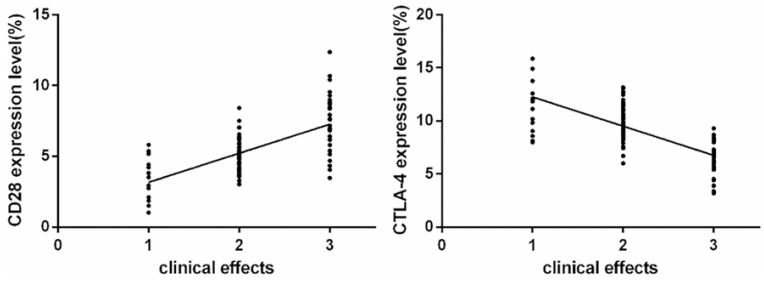

Figure 4. Correlation of CD28 and CTLA-4 levels with clinical efficacy of hemophilia in the RG. CD28 level was remarkably positively correlated with the clinical effects, and the better the treatment effect, the higher the CD28. CTLA-4 level was notably negatively correlated with the clinical effects, and the better the treatment effect was, the lower the CTLA-4 was. Note: 1 indicates ineffective, 2 indicates improved, and 3 indicates markedly effective.

Table 2. Comparison of CD28 and CTLA-4 levels in the effective and ineffective groups (x±s)

	Effective group (n=97)	Ineffective group (n=17)	t	P
CD28 (%)	6.01±1.38	4.03±1.27	5.52	0.00
CTLA-4 (%)	8.62±1.73	11.14±2.53	5.14	0.00

and the combined detection enjoyed the highest sensitivity (94.12%) and the highest AUC (0.90), as shown in **Table 4** and **Figure 6**.

Discussion

At present, the survival time of hemophilia patients has been prolonged due to the continuous improvement of hemophilia nursing and advances in technology [15]. The type and frequency of bleeding vary with age and are affected by co-morbidity and co-medication in elderly patients. Hypertension is the most common complication in patients [16]. As outlined by the European Association for Hemophilia and Allied Diseases (EAHAD) and the World Federation of Hemophilia, the optimal care for hemophilia is established and implemented through a well-concerted plan under the guidance of settled principles and priorities [17]. In recent years, coagulation products have enabled patients with severe hemophilia to live a normal life [18], but a series of problems have also ensued. First of all, the formation of the inhibitory autoantibody against coagulation factor VIII is the main complication of severe hemophilia A treatment [19]. Plus that in most children with severe hemophilia, the age of optimal treatment is delayed due to the lack of a positive family history of hemophilia, making it paramount for medical staff to have a general understanding of the clinical manifestations of patients, as a correct diagnosis is a prerequisite for progress [20]. It also urges us to constantly explore the early clinical diagnosis of hemophilia and the factors that can be used to predict the development of hemophilia.

Compared with the CG, CD28 was significantly lower while CTLA-4 was significantly higher in the RG before treatment, which is related to the fact that CD28 and CTLA-4 are homologous T cell receptors that can produce two opposite immune signals needed for activation and inactivation of T cells respectively [21]. In this study, both CD28 and CTLA-4 were abnormally expressed in hemophilia patients, indicating that the two could be used as factors for early diagnosis of hemophilia. Studies have shown that CD28 is the main costimulatory receptor that promotes the full activation of immature T cells [22], while CTLA-4 is an inhibi-

Table 3. Comparison of the value of single and combined detection of CD28 and CTLA-4 in the diagnosis of hemophilia

Items	Sensitivity	Specificity	Youden index	Optimal threshold	AUC	P	95% CI	
							Upper bound	Lower bound
CD28	72.50%	81.58%	0.03	>5.27	0.82	<0.0001	0.87	0.76
CTLA-4	85.83%	71.05%	0.03	<9.45	0.82	<0.0001	0.88	0.77
CD28+CTLA-4	74.16%	89.47%	0.02	-	0.91	<0.0001	0.94	0.87

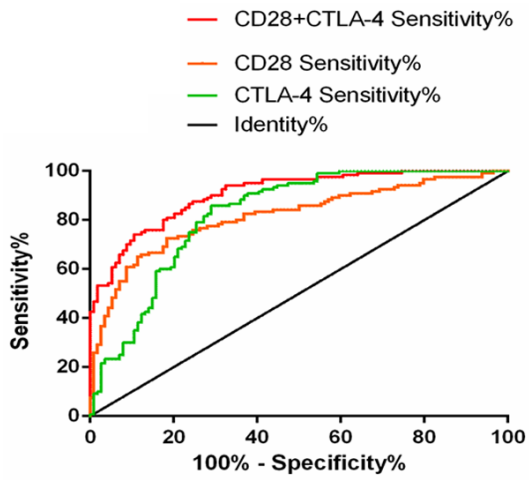

Figure 5. ROC curves of single and combined detection of CD28 and CTLA-4 in the two groups before treatment. The order of sensitivity from high to low was CTLA-4, combined detection and CD28, and the specificity from high to low was combined detection, CD28, CTLA-4. The highest AUC was found in the combined detection, which was up to 0.91.

tory receptor of the CD28 immunoglobulin subfamily. Due to its inhibitory effect, CTLA-4 is a key regulator of T-cell homeostasis and self-tolerance [23], which is also recognized as a key immune checkpoint and a new target for autoimmunity and cancer therapy [24]. Combined with the fact that CD28 increased remarkably and CTLA-4 decreased significantly in the RG after treatment, suggesting that both CD28 and CTLA-4 could serve as factors for early diagnosis and prognosis of hemophilia. Multiple studies have revealed that CD28 and CTLA-4 are related to the degree and efficacy of a wide spectrum of diseases. For example, T cell costimulatory signals modified by anti-CD19 chimeric antigen receptors containing CD28 show great potential in the treatment of acute lymphoblastic leukemia [25]. CD28 is also significantly associated with the risk of tuberculosis [26], and its varying levels in lung adenocarcinoma shows significantly different prognosis and tumor immunity [27]. There is a strong positive correlation between CTLA-4 and the specific marker gene expression of immune cells, and the prognosis of glioma is related to the increase of CTLA-4 expression level [28]. In this study, we identified a significant negative correlation between CD28 and the degree of bleeding, a marked positive correlation between CD28 and clinical curative effect, a significant positive correlation between CTLA-4 and the degree of bleeding, and a notable negative correlation between CTLA-4 and clinical curative effect, indicating that CD28 and CTLA-4 may be vital indicators for early prediction and prognosis of hemophilia. The most important treatment for complications of hemophilia is the development of neutralizing antibodies (inhibitors) against exogenously administered factor VIII. The only effective therapy to eradicate these inhibitors is based on immunity, while the induction of peripheral tolerance of factor VIII involves the inability to induce antigen-specific effector T cells, the induction of regulatory T cells and the formation of anti-idiotypic antibodies [29], indicating the treatment of hemophilia is inseparable from human T cells. Therefore, through single and combined detection of CD28 and CTLA-4 before treatment, we further assessed their diagnostic value in hemophilia patients in RG and VG, and evaluated their prognostic value in hemophilia patients in effective group and ineffective group. ROC curves were further drawn according to the sensitivity and specificity of the single and combined tests. The higher the AUC value is, the higher the diagnostic value is [30]. In the present study, although the single detection of CD28 and CTLA-4 showed some certain diagnostic and prognostic value, the AUCs of the combined tests (0.91, 0.90) were the highest, which indicated that the combined detection was better than the single detection.

In this paper, the expression levels of CD28 and CTLA-4 on the surface of peripheral blood

Table 4. The value of single and combined detection of CD28 and CTLA-4 in predicting the clinical effects of hemophilia

Items	Sensitivity	Specificity	Youden index	Optimal threshold	AUC	P	95% CI	
							Upper bound	Lower bound
CD28	88.66%	85.94%	0.06	<4.27	0.82	<0.0001	0.94	0.70
CTLA-4	70.59%	69.07%	0.08	>9.42	0.72	0.0043	0.87	0.57
CD28+CTLA-4	94.12%	77.32%	0.03	-	0.90	<0.0001	0.96	0.84

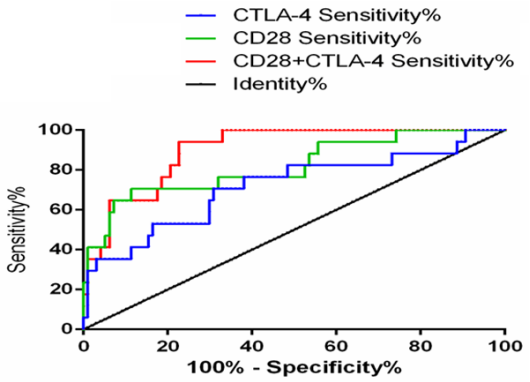

Figure 6. ROC curves of single and combined detection of CD28 and CTLA-4 in the effective group and the ineffective group before treatment. The order of sensitivity from high to low was combined detection, CD28 and CTLA-4, and the specificity from high to low was CD28, combined detection, CTLA-4. The AUC of combined detection was the highest, which was up to 0.90.

T cells were measured individually and jointly to explore their predictive and prognostic significance in hemophilia. However, this study only judged the clinical effects for a limited time, but did not follow up hemophilia patients for a long time to observe their prognosis to determine whether CD28 and CTLA-4 could be used as the prediction of long-term efficacy, so there were certain limitations. Apart from that, the very mechanism of CD28 and CTLA-4 in the occurrence and development of hemophilia remains to be further studied. In view of the above shortcomings, a more complete experimental analysis will be carried out as soon as possible to obtain the best experimental results in the future research.

In conclusion, CD28 decreased remarkably on the surface of T cells in peripheral blood of hemophilia patients, while CTLA-4 increased dramatically, both of which could be used for early clinical diagnosis and prediction of disease progression in patients with hemophilia.

Disclosure of conflict of interest

None.

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