ANALYSIS OF T CELL IMMUNE FUNCTION OF 16 PATIENTS WITH CHRONIC THROMBOEMBOLIC PULMONARY HYPERTENSION

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ABSTRACT

***Aims**: The aim of this study is to observe the T cell immune function in patients with chronic thromboembolic pulmonary hypertension (CTEPH).*

***Materials and methods**: Expressions of T cell surface antigens including CD3+, CD4+, CD8+ and CD4+/CD8+ ratio were examined in 16 patients with CTEPH, with a follow-up of 1 year. Follow-up data were acquired from 9 patients (56%). mRNA expression of T cell immunity related genes was measured by whole human genome microarray in peripheral blood mononuclear cells (PBMCs) of some patients and 20 controls.*

***Results**: Of 16 patients, 7 (44%) had abnormal CD3+ expression (2 were increased, 5 were decreased), 5 (31%) had increased CD4+ expression, 8 (50%) had decreased CD8+ expression, and 10 had abnormal CD4+/CD8+ ratio (8 were increased, 2 were decreased). Of 9 patients followed up, 7 (78%) had decreased CD3+ expression, 5 (55%) had abnormal CD4+ expression (1 was increased, 4 were decreased), 6 (67%) had decreased CD8+ expression, and 6 had increased CD4+/CD8+ ratio. When compared with controls, the mRNA expressions of CD3D, CD3G, GZMA, GZMB and ZAP70 were markedly down-regulated in 10 CTEPH patients ($P<0.05$).*

***Conclusion**: The T cell functions of antigen recognition, signal transduction and killing pathogens in patients with CTEPH were reduced, and T cell immune dysfunction may play an important role in the occurrence and development of CTEPH.*

***Key words**: Chronic thromboembolic pulmonary hypertension, T cell immune function, pulmonary embolism, RNA messenger.*

***Received** June 18, 2014; **Accepted** October 02, 2014*

Introduction

Chronic thromboembolic pulmonary hypertension (CTEPH) is one of the leading causes of severe pulmonary vascular disease[(1)]. It has traditionally been believed that CTEPH is the extended stage of acute pulmonary embolism (APE)[(2)]. Pengo et al. have reported that the incidence of CTEPH after APE was 1.0% at 6 months, 3.1% at 1 year and 3.8% at 2 years[(3)]. In 2011, the author's team has reported that patients with APE had reduced immune function[(4)], which indicates that immune dysfunction may be related to the occurrence of VTE. However, the pathogenesis of CTEPH is still unclear and there is no history of symptomatic venous thromboembolism (VTE) in 31-42% of the patients diagnosed with CTEPH[(5-7).] Based on the genomic results of our previous study[(8)] that T cells' function including antigen recognition, signal transduction and cytotoxicity was impaired in VTE patients at the gene expression levels, this study aimed to investigate the T cell immune function in 16 patients with CTEPH at both gene expression and protein levels and to find out the possible pathogenesis of CTEPH.

Materials and methods

Subjects

A total of sixteen patients (four male, twelve female) with CTEPH diagnosed in Tongji Hospital from 2007-2012 were included in this study, with a

mean age of 63±19 years old (11-87 years old). The diagnosis of CTEPH was based on the criteria developed by the American College of Chest Physicians and American Heart Association respectively[9, 10]:

(1) systolic pulmonary artery pressures assessed by Doppler echocardiography ≥ 40 mmHg or mean pulmonary artery pressures assessed by right heart catheterization > 25 mmHg; abnormal pulmonary ventilation-perfusion scan, pulmonary CT angiography and selective pulmonary angiography;

(2) clinical diagnosis: risk factors of PE or diagnosed with VTE, exertional dyspnea lasting for more than six months, other cardiopulmonary diseases excluded by electrocardiography and chest X-ray. All patients except one had exertional dyspnea when diagnosed with CTEPH. Five patients (31%) were in New York Heart Association (NYHA) class II, nine (56%) in NYHA class III, and two (13%) in NYHA class IV. The World Health Organization (WHO) classification of pulmonary hypertension of all patients was the same as the NYHA classification of them. Only two patients (13%) had positive D-dimer (>0.3mg/l). Doppler echocardiography was performed in all patients, and the systolic pulmonary arterial pressure ranged from 44 to 120 mmHg (mean 74.6±22.8).

Whole human genome microarray of peripheral blood mononuclear cells (PBMCs) was done in 10 patients (3 males and 7 females with a mean age of 66±12 years [range: 43-84 year]). In addition, 20 hypertension patients matched in age and gender with CTEPH patients were recruited from the Department of Cardiology (6 males and 14 females with a mean age of 63±14 years [range: 44-86 years]). There were no marked differences in age and gender between two groups (P>0.05). Moreover, PE, deep venous thrombosis, CTEPH and congenital coagulopathy were excluded, and the D-dimer was <0.3mg/L.

During 1 year follow-up (one patient had been enrolled for less than four months), 3 patients died and 4 lost to follow up. Among 9 patients followed up, there was only patient (11%) who had positive D-dimer (>0.3mg/l). Doppler echocardiography was also performed in these 9 patients, and the systolic pulmonary arterial pressure ranged from 32 to 89 mmHg (mean 54.1±17.4). Eight patients (89%) were in NYHA class II, one (11%) in NYHA class III. The original NYHA classification of 3 patients who died was III-IV. WHO classification of pulmonary hypertension of all patients was the same as their NYHA classification.

Malignancies and autoimmune diseases were excluded in all patients. The Ethics Committee of Tongji Hospital approved this study. Informed consent was obtained from each patient before study.

Whole human genome microarray

Instruments and sample collection: Agilent human genome-wide oligonucleotide chip (G4112A; Agilent, USA) was used and included 44290 spots (41675 genes or transcripts, 314 negative quality control spots, 1942 positive quality control spots, 359 blank spots). More than 70% of genes in this chip have clear function. Venous blood (5 ml) was obtained from patients in two groups and anti-coagulated with ethylene diamine tetraacetic acid.

Extraction and purification of total RNA: Erythrocyte lysis buffer (Qiagen, Hilden, Germany) was used to separate PBMC. Total RNA was extracted from PBMCs with TRIzol (Invitrogen, Carlsbad, USA). Detection of total RNA was done by agarose gel electrophoresis and Lab-on-chip PCR, which suggests RNA with high purity. QIAGEN RNeasy column was used to purify the extracted RNA, and DNase was employed to remove genomic DNA.

Detection of gene expression: Indirect method was used to label samples. Total RNA was reverse transcribed into cDNA followed by purification. Cy3 conjugated cRNA and fluorescence conjugated cRNA were used to hybridize with chip at 60°C at a constant rotation rate of 10 r/min for 17 h. Then, the products were washed with Agilent gene expression elution buffer. The row data were obtained with Agilent scanner and supporting Feature Extraction. Robust Multi-array Average (RMA) was used to standardize these raw data. The standardized data were used for further analysis.

Real-time quantitative PCR: Simple random sampling was performed to randomly select 3 differentially expressed genes (CCL3L3, ZNF683, LBA1) and house-keeping gene (GAPDH) for RT-PCR aiming to confirm the results from microarray.

Examination of differentiation antigens on T cells

2ml of fasting venous blood was obtained from all patients in the morning, and then the sample was added into the ET tube. The differentiation antigens on T cells including CD3+, CD4+, CD8+

and CD4+/CD8+ ratio in all patients were examined by BECKMANCOULTER EPICS XL-II flow cytometer.

Statistical analysis

Statistical analysis was done with Statistical Product and Service Solutions (SPSS) version 12.0 for Windows (IBM Ltd.). Quantitative data were expressed as mean ± standard deviation (x±s). Comparisons of standardized data were done with t test between two groups. A value of $P<0.05$ was considered statistically significant.

Results

mRNA expression of T cell immunity related genes in two groups

When compared with controls, the mRNA expressions of CD247, CD3D, CD3G, GZMA, GZMB and ZAP70 were reduced markedly in PBMCs of CTEPH patients ($P<0.05$ for all) (Table 1).

Group	Case	CD3D mRNA	CD3G mRNA	GZMA mRNA	GZMB mRNA	ZAP70 mRNA
Control	20	14.3820±0.4857	14.1246±0.6011	15.5305±0.4624	16.4553±0.5055	14.9229±0.4133
CTEPH	10	13.0376±0.5686	12.8259±0.7825	14.1923±0.5894	15.2647±0.7059	14.1762±0.4721
P		0.0043	0.0019	0.0048	0.0117	0.007

Table 1: mRNA expression of T cell immunity related genes in PBMCs of CTEPH patients and controls (x±s).
Footnotes: PBMC = peripheral blood mononuclear cells; CTEPH = chronic thromboembolic pulmonary hypertension

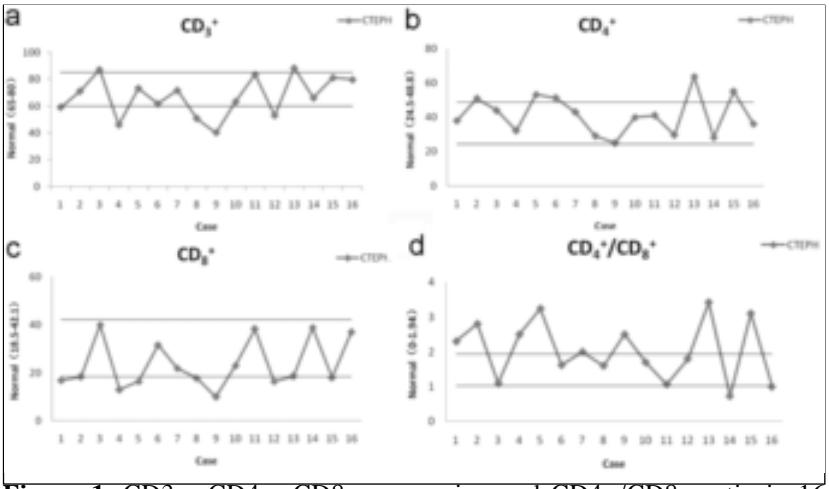

Figure 1: CD3+, CD4+, CD8+ expression and CD4+/CD8+ ratio in 16 patients with chronic thromboembolic pulmonary hypertension. (a) 7 (44%) patients had abnormal CD3+ expression (2 were increased, 5 were decreased); (b) 5 (31%) had increased CD4+ expression; (c) 8 (50%) had decreased CD8+ expression; (d) 10 had abnormal CD4+/CD8+ ratio (8 were increased, 2 were decreased).

Expression of T cell differentiation antigens in 16 patients with CTEPH

The results showed that the differentiation antigens on T cells including CD3+, CD4+, CD8+ and CD4+/CD8+ ratio in 16 patients with CTEPH were detected (Fig. 1A-D, Table 2). Among them, 7 (44%) had abnormal CD3+ expression (2 were increased, 5 were decreased), 5 (31%) had increased CD4+ expression, 8 (50%) had decreased CD8+ expression, and 10 had abnormal CD4+/CD8+ ratio (8 were increased, 2 were decreased).

Expression of T cell differentiation antigens in 9 patients with CTEPH at 1 year follow-up

The differentiation antigens on T cells including CD3+, CD4+, CD8+ and CD4+/CD8+ ratio were examined in 9 patients with CTEPH followed up (Fig. 2A-D, Table 3). Among them, there were 7 (78%) with decreased CD3+ expression, 5 (55%) with abnormal CD4+ expression (1 was increased, 4 were decreased), 6 (67%) with decreased CD8+ expression, and 6 with increased CD4+/CD8+ ratio.

Discussion

It is generally accepted that CTEPH is initiated by thromboembolism. However, the concept that the occurrence of CTEPH may be related to inflammation and infection has been proposed recently(11, 12). Our previous genomic studies have shown that the immune function of T cells and T cell receptor complex was reduced in patients with PE. When antigens are recognized by T cell receptors (TCRs), T cells are stimulated and transferred into effector T cells which kill target cells directly or indirectly by releasing cytokines. T cells play a central role in adaptive immunity.

CD3 is an important molecule on the surface of T cells, and composed of 5 types of peptide chains. CD3 can bind to TCR to form TCR-CD3 complex. Lacking of either δ chain or γ chain may significantly influence the generation and activation of T cells. T cells could be divided into CD4+ and CD8+ subsets.

	Increased cases, n (%)	Decreased cases, n (%)
CD3+	2/16 (13)	5/16 (31)
CD4+	5/16 (31)	0/16 (0)
CD8+	0/16 (0)	8/16 (50)
CD4+/CD8+	8/16 (50)	2/16 (13)

Table 2: Expression of T cell differentiation antigens in 16 patients with CTEPH.
Footnotes: CTEPH = chronic thromboembolic pulmonary hypertension

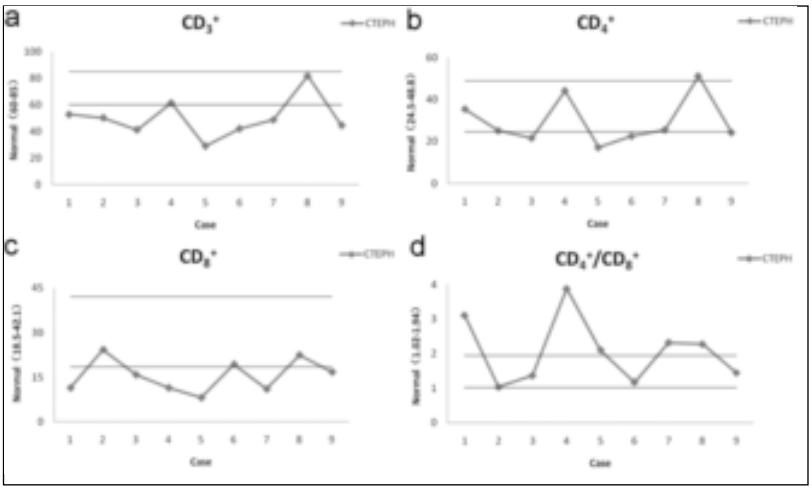

Figure 2. CD3+, CD4+, CD8+ expression and CD4+/CD8+ ratio in 9 patients with chronic thromboembolic pulmonary hypertension at 1 year follow-up. There were 7 (78%) patients with decreased CD3+ expression (a), 5 (55%) with abnormal CD4+ expression (1 was increased, 4 were decreased) (b), 6 (67%) with decreased CD8+ expression (c), and 6 with increased CD4+/CD8+ ratio (d).

	Increased cases, n (%)	Decreased cases, n (%)
CD3+	0/9 (0)	7/9 (78)
CD4+	1/9 (11)	4/9 (44)
CD8+	0/9 (0)	6/9 (67)
CD4+/CD8+	5/9 (56)	0/9 (0)

Table 3: Expression of T cell differentiation antigens in 9 patients with CTEPH at 1 year follow-up.
Footnotes: CTEPH = chronic thromboembolic pulmonary hypertension

CD8+ T cells destroy target cells infected with virus or other intracellular pathogens and mutant cells in vivo, whereas CD4+ T cells regulate and assist in the immune response.

Our results showed the expression of both CD3D (δ chain) and CD3G (γ chain) was significantly reduced in PMBCs of CTEPH patients when compared with controls. This suggests that the transformation of mature T cells into effector T cells is inhibited, and structure and function of TCR-CD3 complex are also influenced. In addition, of 16 patients, approximately half had abnormal CD3+ expression. At 1 year follow-up, 8 (89%) patients had decreased CD3+, which further indicates that the T cell activation is significantly compromised and the T cell immunity is injured in CTEPH patients.

ZAP-70 plays an important role in early signal transduction required for TCR-CD3 complex mediated T cell activation. ZAP-70 gene mutation may cause selective T-cell deficiency that is characterized by lacking of CD+8T cells. Our results showed the ZAP-70 mRNA expression in T cells of CTEPH patients reduced markedly, suggesting the suppressed T cell activation, especially the cytotoxic T lymphocytes (CTL) activation. In addition, 8 (50%) patients had decreased CD8+ expression, 8 (50%) had increased CD4+/CD8+ ratio, which further confirmed that the CTL activation is suppressed in CTEPH patients. At 1 year follow-up, 8 (89%) patients had decreased CD3+ or CD8+ expression, 5 (56%) had increased CD4+/CD8+ ratio. Our findings indicate sustained T cell immune dysfunction in patients with CTEPH.

Granzyme is mainly derived from cytoplasmic granules secreted by CTL and important for the killing of cancer cells and virus-infected cells by CTL. Granzyme has been regarded as a marker for CTL activation. GzmA and GzmB are the major members of granzyme family. Our results showed the mRNA expressions of GzmA and GzmB in CTEPH patients reduced dramatically when compared with the controls, suggesting that the ability of CTL to release granzyme is significantly compromised when compared with controls. The reduced CTL activation may decrease the ability of T cells to kill virus and cancer cells.

On admission, 31% of 16 patients CTEPH had increased CD4 cells. T cells with delayed-type hypersensitivity (TDTH/TD) may secrete cytokines after activation, which then recruit macrophages into the injured sites resulting in their activation and formation of chronic inflammation. Currently, in CTEPH patients, vasoconstrictor response medi-

ated by a variety of inflammatory cytokines, is a major cause of occurrence and deterioration of CTEPH. In 1-year follow up, 44% of patients had reduced CD4+ cells, which suggests that the cellular immunity presents with decreasing tendency with the deterioration of disease condition.

It is generally believed that there are four major causes of T cell immune dysfunction, 1) infection, such as virus infection; 2) tumor; 3) severe malnutrition; 4) iatrogenic factors, such as use of immunosuppressive drugs. In our study, all the causes were excluded except the first one. The author's team has reported that through an electron microscope, virus-like microorganisms were identified in the cytoplasm of the T lymphocytes of a patient with pulmonary hypertension in our study (13). Thus, it is speculated that virus infection may be an etiologic factor of CTEPH. Smeeth et al. have reported that acute infections were related to an increased risk of VTE (14). We have reported that VTE was found in multiple organs from a patient who died of severe acute respiratory syndrome (15). All the studies above indicate that virus infection is related to the occurrence of VTE, which is consistent with the results of this study.

The reduction of CD3+ expression indicates that the T cell function of antigen recognition and signal transduction decreased in CTEPH patients. The reduction of CD8+ expression suggests that the T cell function of killing pathogens decreased in CTEPH patients. Therefore, with the decrease T cell function of antigen recognition and activation, the T cell immune function may lose the ability of killing certain virus that causes the body infection and lead to recurrent or persistent viral infection.

If the inflammation cannot be controlled, endothelial damage and vascular remodeling would be induced, which promotes the progress of the disease.

The limitation of this study is a lack of patients' number. Future studies of lager scale should be conducted. Besides, the mechanisms how T cell immunity dysfunction could lead to CTEPH should be investigated deeply in the future.

The results of our study indicate that the T cell immune dysfunction is closely related to the occurrence of CTEPH, and may also play an important role in the progress of CTEPH. Moreover, both the results of early stage and one year follow-up suggest that T cell immune dysfunction may contribute to CTEPH.

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Acknowledgements
This study was supported by Project of Shanghai Municipal Health Bureau (No: 2010108), Key projection of Shanghai Science and Technology Commission (No: 11411951400) and Project for Youth of Shanghai Municipal Health Bureau (No: 2009Y071).

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