Int J Clin Exp Pathol 2015;8(7):8244-8251
www.ijcep.com /ISSN:1936-2625/IJCEP0009692

Original Article

Compromised natural killer cells in pulmonary embolism

Xiaoyu Zhang[1*], Qiang Wang[2*], Yuqin Shen[2], Haoming Song[2], Zhu Gong[2], Lemin Wang[2]

[1]Department of Rheumatology, Tongji Hospital of Tongji University, Shanghai 200065, China; [2]Department of Cardiology, Tongji Hospital of Tongji University, Shanghai 200065, China. [*]Equal contributors.

Received April 28, 2015; Accepted June 22, 2015; Epub July 1, 2015; Published July 15, 2015

Abstract: Objective: The high morbidity, mortality and misdiagnosis rate render pulmonary embolism (PE) as a worldwide health problem. However, the etiology and pathogenesis of this disease have not been well characterized. Increasing studies indicate infection and immunity play a crucial role in PE. Natural killer (NK) cells act as a bridge between the innate immune and acquired immune. This study aimed to investigate the possible function of NK cells in PE. Methods: Human cDNA microarray analysis was employed to detect genes associated with NK cells in peripheral blood mononuclear cells (PBMCs). Random variance model corrected *t*-test was used for statistical analysis of differential gene expression. Flow cytometry was performed to detect the CD16+CD56+ NK cells. Results: In the present study, based on gene expression microarray analysis, we showed four inhibitory receptors (KLRB1, KLRD1, KLRF1, KLRG1) and four activating receptors (KLRC1, KLRC3, KLRK1 and NCR1) on NK cells were remarkably down-regulated and the cytological experiment demonstrated the proportion of CD16+CD56+ NK cells among PBMCs decreased in the PE group. Conclusions: We confirmed the presence of reduced expression of critical activating as well as inhibitory NK cell receptors and low proportion of CD16+CD56+ NK cells in PE. The consistence between genomic and cytological examination suggests compromised NK cells may contribute to the pathogenesis of PE.

Keywords: Pulmonary embolism, gene, natural killer cell

Introduction

Venous thromboembolism (VTE) represents a spectrum of conditions that include pulmonary embolism (PE), chronic thromboembolic pulmonary hypertension (CTEPH) and deep venous thrombosis (DVT). The high morbidity, mortality and misdiagnosis rate render VTE as a worldwide health problem [1]. The American College of Chest Physicians has recommended nine editions of the guideline for the diagnosis and prevention of VTE since 1995 and also proposed the concept of risk stratification for prophylaxis of VTE [2]. However, the incidence of symptomatic VTE is increasing gradually instead of reducing, partly because the etiology and pathogenesis of this disease have not been well characterized [3].

It has been demonstrated that genetic and environmental factors are involved in the development of VTE and increasing studies indicate that infection and immunity play a crucial role in the pathogenesis of PE. In 2006, Smeeth et al [4] reported infection was associated with an increased VTE risk. The association was strongest within the first 2 weeks after infection onset. In 2012, Schmidt et al retrospectively investigated 15009 cases of VTE and showed that risk for VTE was increased two-fold in patients with infection, especially within two weeks of infection, gradually declining thereafter [5]. Since 2004, we have been engaged in basic and clinical investigation of VTE. We observed virus-like microorganism [6], rod-shaped bacteria like microorganisms [7] and SARS coronavirus infection [8] in patients with VTE. All these findings indicate that onset of VTE is closely related with microorganisms infections. In addition, we discovered the impaired cytotoxic activity of T cells [9] and compromised complement system in VTE patients [10], strongly supporting an involvement of the immune system in the pathogenesis of VTE. Natural killer (NK) cells are a type of cytotoxic lymphocyte critical to the innate immune sys-

tem. What's more, numerous experiments have worked to demonstrate their ability to readily adjust to the immediate environment and formulate antigen-specific immunological memory, fundamental for responding to secondary infections with the same antigen. On the basis of previous findings, this study was undertaken to detect the mRNA expression levels of NK cells in PBMCs isolated from PE patients and controls by the whole human genome microarray and Gene Ontology analysis. In addition, flow cytometry was performed to investigate the changes in NK cells in PE patients, which aimed to validate the results from genome analysis.

Materials and methods

Subjects

All subjects were from Tongji Hospital of Tongji University. The diagnostic criteria for pulmonary embolism were any two of the following: (1) selective pulmonary angiography showing pulmonary artery occlusion or filling defect; (2) pulmonary ventilation-perfusion scan showing single or multiple perfusion defect, normal or abnormal ventilation, and ventilation-perfusion mismatch; (3) clinical diagnosis: risk factors for PE are present and other cardiovascular diseases may be excluded by clinical performance, electrocardiogram and chest film; arterial blood gas analysis, D-dimer detection, echocardiography and/or chest computed tomography support PE diagnosis. The diagnosis of DVT was based on the criteria previously reported [11] and the criteria for CTEPH was used in accordance with the diagnosis of CTEPH [12]. Malignancies, use of immunosuppresants or autoimmune diseases were excluded in all subjects. Healthy controls underwent physical examination before they were recruited from the outpatient clinic. The present study was approved by the Ethics Committee of Tongji Hospital and informed consent was obtained before study.

Genomic study

Twenty PE inpatients and 20 controls were randomly selected in Cardiology Department, Tongji Hospital of Tongji University. In the PE group, there were 11 males and 9 females, with a mean age of 70±14 years (44~89 years). There were 13 patients with acute PE and 7 with CTEPH. The pulmonary artery pressure was 50-108 mmHg in CTEPH patients. In the control group, 20 patients (11 males and 9 females) with a mean age of 72±14 years (44~91 years) were enrolled during the same period. No significant difference in age was found between PE patients and controls ($P > 0.05$).

Cellular immunology

A total of 41 clinically proven VTE patients (31 with APE, 9 with DVT and 10 with CTEPH) aged 67±12 years (31~88 years) were recruited from Tongji Hospital, including 18 males and 23 females. The proportion of CD16+CD56+ NK cells in VTE patients were all compared with the detection intervals of normal population (8.6-21.1%).

Extraction of total RNA

A total of 5 ml of venous EDTA anti-coagulated blood was obtained from patients of both groups and mononuclear cells were isolated by density gradient centrifugation. Red blood cell lysis buffer (Qiagen, Hilden, Germany) was used to isolate mononuclear cells and total RNA was extracted from mononuclear cells with TRIzol (Invitrogen, Carlsbad, USA) followed by purification with RNeasy column (QIAGEN). Treatment with DNase was performed to avoid the influence by genomic DNA. Quantification of extracted RNA was performed with Nanodrop ND-1000 spectrophotometer (Nanodrop Technology, Cambridge, UK).

Gene expression profiling

Agilent G4112A Whole Human Genome Oligo Microarrays were purchased from Agilent (USA). A microarray is composed of 44,290 spots including 41675 genes or transcripts, 314 negative control spots, 1924 positive control spots and 359 blank spots. The functions of more than 70% of genes in the microarray have been known. All patients of both groups were subjected to microarray analysis.

Sample marking and hybridization

Indirect approach was applied to mark samples. About 1 μg of total RNA was reversely transcribed into double strand cDNA. After purification, *in vitro* amplification was performed with Agilent Low RNA Input Linear Amplification

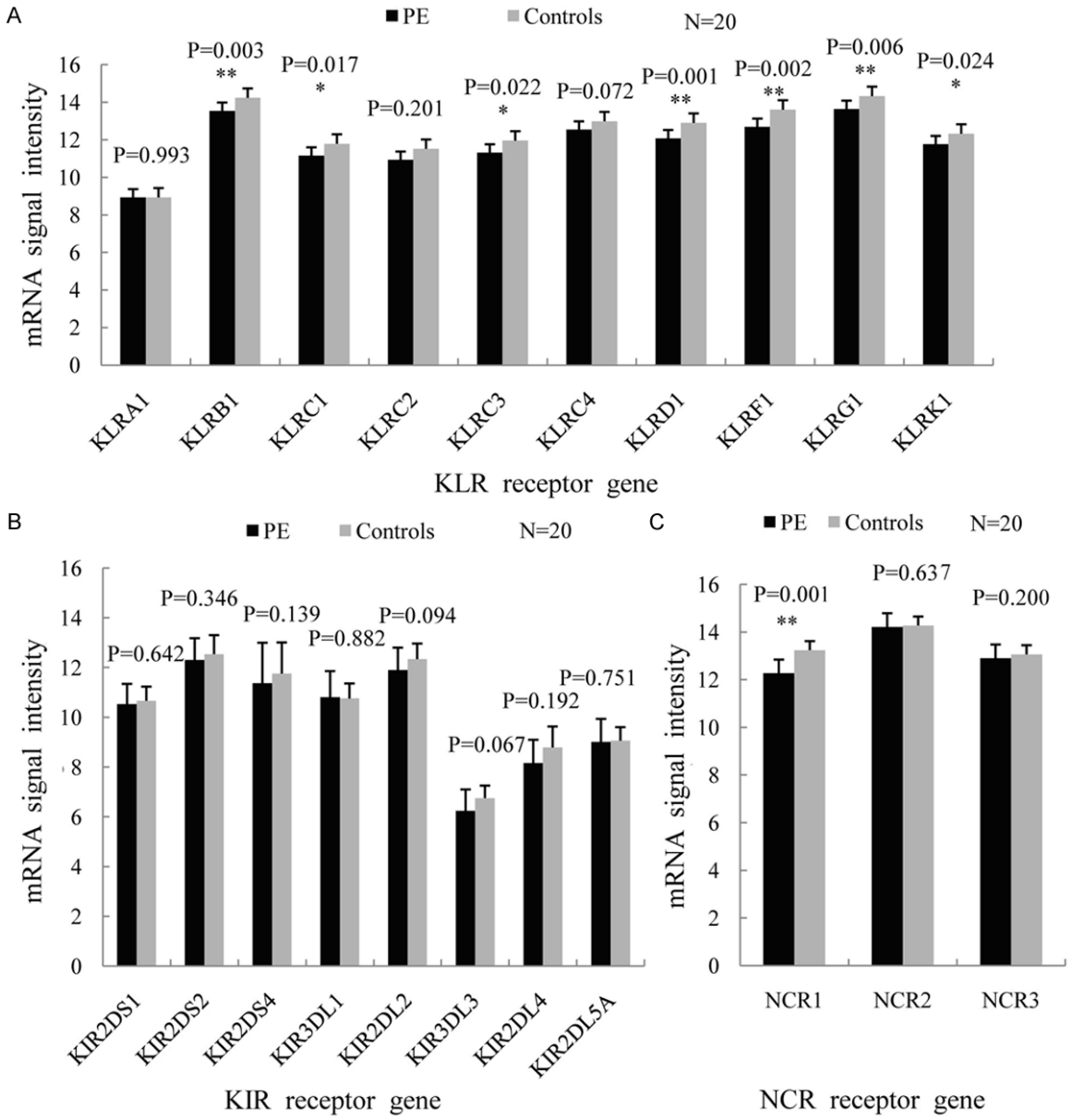

Figure 1. Microarray results of NK cell surface receptors. A. KLR receptors were down-regulated totally in PE group. The difference of KLRC1, KLRC3 and KLRK1 showed statistical significance (*P<0.05), and KLRB1, KLRD1, KLRF1 and KLRG1 were remarkably down-regulated (**P<0.01). B. The mRNA expression of KIR receptors was lower than that of controls consistently, but the difference had no statistical significance. C. NCR receptors were down-regulated and NCR1 was remarkably down-regulated (**P<0.01).

Kit (Agilent, Pal alto, USA) and modified UTP [aaUTP, 5-(3-aminoally1)-UTP] was used to replace UTP. The integrated aaUTP can interact with Cy3 NHS ester forming fluorescent products which are then used for hybridization. The integration rate of fluorescence can be determined with a NanodropND-1000 spectrophotometer. Then, hybridization mixture was prepared with Agilent oligonucleotide microarray in situ hybridization plus kit. About 750 ng of fluorescent products were fragmented at 60°C and hybridization was conducted in Human Whole-Genome 60-mer oligo-chips (G4112F, Agilent Technologies) at 60°C for 17 h at 10 rpm. After hybridization, the chips were washed with Agilent Gene Expression Wash Buffer according to manufacturer's instructions. Original signals were obtained Agilent scanner and Feature Extraction software. The standardization of original signals was carried out with RMA stan-

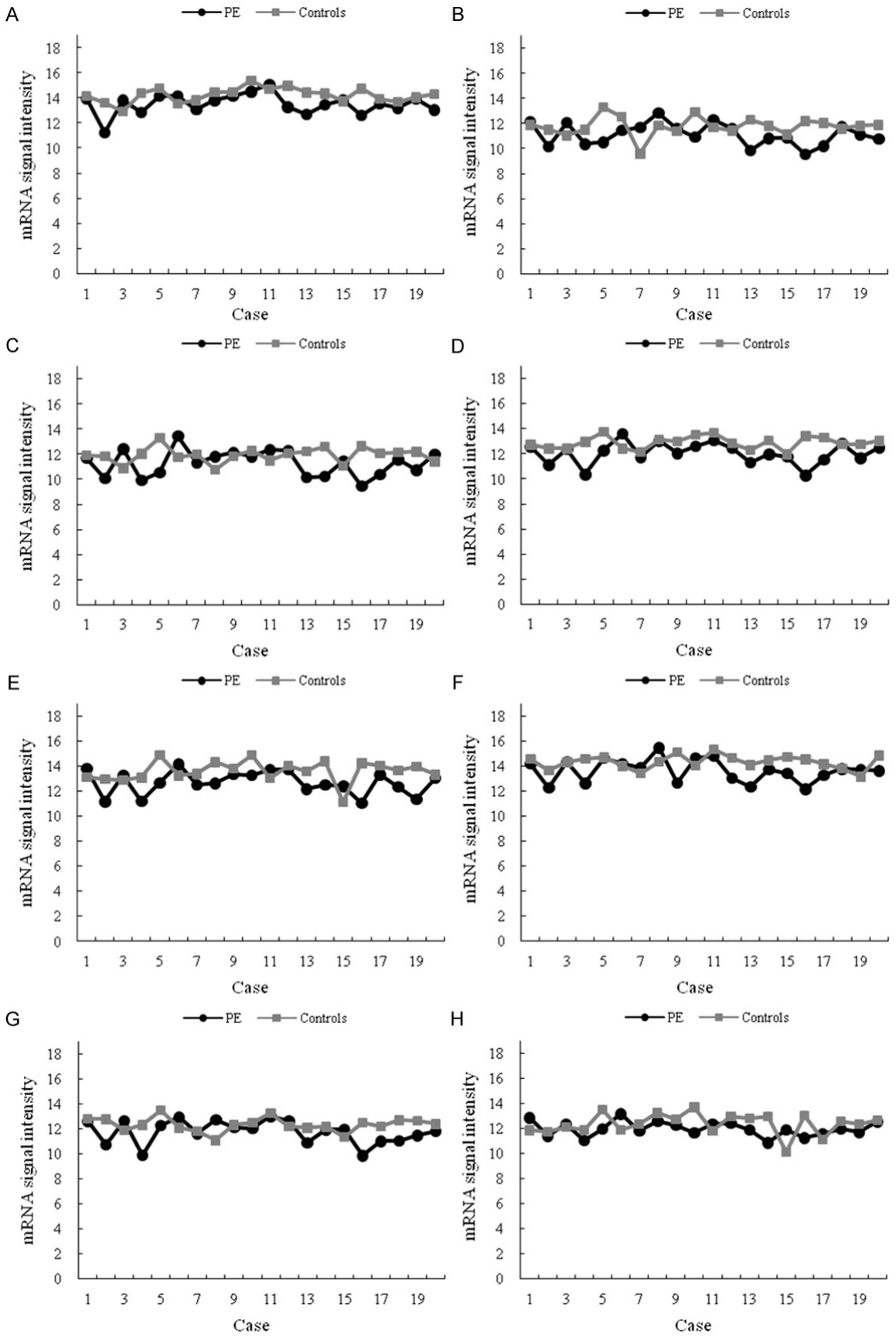
A
B
C
D
E
F
G
H
PE
Controls
mRNA signal intensity
Case
0
2
4
6
8
10
12
14
16
18
1
3
5
7
9
11
13
15
17
19

Figure 2. Microarray results of remarkably down-regulated receptors of NK cells. Crucial receptors including KLRB1 (A), KLRC1 (B), KLRC3(C), KLRD1 (D), KLRF1 (E), KLRG1 (F), KLRK1 (G) and NCR1 (H) were significantly down-regulated.

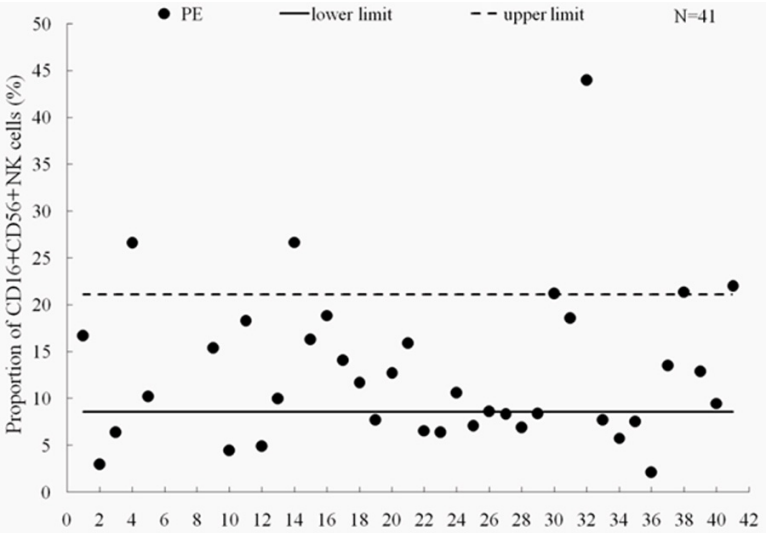

Figure 3. Analysis of peripheral CD16+CD56+ NK cells proportion by flow cytometry. The CD16+CD56+ NK cells were decreased in 16 out of 41 patients compared with the detection intervals of normal population (8.6-21.1%), indicating that the proportion of activated NK cells declined.

dardized method and standardized signal values were used for screening of differentially expressed genes.

RT-PCR

The spots in the microarray were randomly selected and their expressions were confirmed by RT-PCR. Among genes with differential expressions, 3 genes were randomly selected and these genes and house-keeping gene (GAPDH) were subjected to RT-PCR. The relative expressions were expressed as the expressions of target genes normalized by that of GAPDH ($2^{-\Delta\Delta Ct}$). Melting curve and $2^{-\Delta\Delta Ct}$ method were used to compare the difference in the expressions between control group and PE group. Results from RT-PCR were consistent with microarray analysis.

Significant differential gene expression analysis

Agilent Feature extraction software was used to collect original data from microarray followed by analysis with robust multichip average (RMA). Gene intensity data between PE group and control group were compared with t test after calibration with a stochastic variance model. Differentially expressed genes were identified from whole genomes. Independent-Samples T Test was used to compare mRNA levels in samples from PE patients and controls. Statistical tests were performed using SPSS 17.0, and *P* values <0.05 were considered significant. Before t test, test for equality of variances was performed, if variances were not equal, t test result would be corrected.

Detection of differentiation antigens on NK cells

Sample collection: the fasting venous blood (2 ml) was collected in the morning and added to the ET tube. Flow cytometry was performed to detect the differentiation antigens on NK cells with BECKMANCOULTER EPICS XL-II flow cytometer. The NK cell marker CD16+CD56+ was detected in 41PE patients.

Results

mRNA expressions of NK cells related genes

NK cells related surface receptors, including killer cell lectin-like receptor (KLR), killer cell immunoglobulin-like receptor (KIR) and natural cytotoxicity receptor (NCR) were uniformly down-regulated. In the PE group, four inhibitory receptors (KLRB1, KLRD1, KLRF1, KLRG1) and four activating receptors (KLRC1, KLRC3, NCR1 and KLRK1) which positively correlate with NK cytotoxicity, were remarkably down-regulated compared with control group (*P*<0.05), while the difference of KIR receptors, KLRC2, KLRC4, NCR2 and NCR3 between two groups had no statistical significance (**Figures 1**, **2**).

Flow cytometry analysis of CD16+CD56+ NK cells

Flow cytometry was performed to detect peripheral blood NK cell surface marker CD16+CD56+. The CD16+CD56+ NK cells were decreased in

16 out of 41 patients (39.02%), suggesting that the proportion of activated NK cells decreased (**Figure 3**).

Discussion

Based on gene expression microarray analysis, we have shown in the present study four inhibitory receptors (KLRB1, KLRD1, KLRF1, KLRG1) and four activating receptors (KLRC1, KLRC3, KLRK1 and NCR1) on NK cells were remarkably down-regulated and the cytological experiment demonstrated the proportion of CD16+CD56+ NK cells among PBMCs reduced in the PE group. The activating and inhibitory receptors of NK cells are key mediators in controlling the immune reaction to viral infection [13]; thus, it is likely that both the low gene expression of NK cell receptors and the reduced proportion of activated NK cells may lead to impairment of NK cell function in this disease. On the other hand, our results showed no significant differences in the expression of KLRA1, KLRC2, KLRC4, NCR2, NCR3 and KIR receptors in this study. Considered together, while it was unknown if they were correlated with each other, these findings indicated that the reduced receptors might play a role at least in part, among the molecules related to NK cell dysfunction in the development of PE.

It is well known that individuals with defective NK cells are susceptible to virus infection and cancer formation. In this study, malignant tumors or use of immunosuppressive drugs were all excluded, so we speculated compromised NK cells in PE patients might be associated with virus infection. Successive scholars demonstrate that individuals who are infected special virus, such as influenza virus [14], herpes simplex virus [15], Epstein-Barr virus [16], cytomegalovirus [17], human immunodeficiency virus [18], have high tendency to VTE this decade. Zhu et al [19] conducted a case-control study involving 1,454 adults enrolled in 11 French centers to investigate whether influenza vaccination reduced the risk of VTE. They concluded that influenza vaccination was associated with a reduced risk of VTE. Viral infections associated with decreased NK cell activity in other human diseases have also been reported. Nakata S et al pointed that the presence of reduced NK activating receptor gene expression and low proportion of NK cells was involved in fulminant type 1 diabetes [20]. Since 2004, we have been engaged in basic and clinical investigation of VTE. In 2010, we reported virus-like microorganisms observed in cytoplasm and intercellular substance of lymphocytes from peripheral venous blood in VTE patients with pulmonary hypertension and T cell immune dysfunction/disorder [6]. We also observed rod-shaped bacteria like microorganisms in apoptotic phagocytes from peripheral venous blood in patients with repeated PE/DVT and T cell immune dysfunction/disorder [7]. Besides, we also reported VTE in multiple organs of a patient who died of SARS, suggesting viral infection is closely related with VTE [8]. All these findings indicate that viral infections associated with decreased NK cell activity contribute to the onset of VTE. Yet most of the molecular mechanisms whereby compromised NK cells interact with virus to promote the occurrence of VTE remain largely undefined. It was reported previously that certain viruses have developed strategies that interfere with NK cell cytotoxicity. For example, through modulation of NK cell receptor ligands on the surface of infected cells or down-regulation of certain activating NK cell receptors to reduce in their cytolytic effectiveness with consequent immune evasion [13]. On basis of our previous findings, we suppose that the impaired NK cells immune function may be the internal cause of susceptibility to acquired VTE, and infection acts as a triggering factor. When the pathogens invade the subjects with NK cell dysfunction, the pathogens cannot be completely removed by the immune system. Thus, patients with compromised NK cell function are susceptible to infection, especially viral infection, which is a high risk factor for VTE. The current study provided a new insight into a novel role for NK cells in the pathogenesis of venous thrombosis from an immune perspective. However, the methodology of this study is relatively simple and understanding the cellular mechanisms whereby NK cells dysfunction contribute to VTE is still an important challenge of biomedical research. Furthermore, Long term follow-up is needed as regards the restoration of pulmonary perfusion, the rate of survival, and the occurrence of new diseases such as cancer in future.

Acknowledgements

This study was supported by “12th five year” National Science and Technology Supporting Program (2011BAI11B16).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Lemin Wang, Department of Cardiology, Tongji Hospital of Tongji University, 389 Xincun Road, Shanghai 200065, P. R. China. Tel: 86-21-66111291; Fax: 86-21-560-50502; E-mail: wanglemin@tongji.edu.cn

References

[1] Cardiovascular Disease Educational and Research Trust; European Venous Forum; North American Thrombosis Forum; International Union of Angiology; Union Internationale du Phlebologie. Prevention and treatment of venous thromboembolism: international consensus statement (guidelines according to scientific evidence). Clin Appl Thromb Hemost 2013; 19: 116-118.

[2] Kahn SR, Lim W, Dunn AS, Cushman M, Dentali F, Akl EA, Cook DJ, Balekian AA, Klein RC, Le H, Schulman S and Murad MH. Prevention of VTE in nonsurgical patients: Antithrombotic Therapy and Prevention of Thrombosis, 9th edition. American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. Chest 2012; 141 Suppl: e195S-226S.

[3] Shackford SR, Rogers FB, Terrien CM, Bouchard P, Ratliff J and Zubis R. A 10-year analysis of venous thromboembolism on the surgical service: the effect of practice guidelines for prophylaxis. Surgery 2008; 144: 3-11.

[4] Smeeth L, Cook C, Thomas S, Hall AJ, Hubbard R and Vallance P. Risk of deep vein thrombosis and pulmonary embolism after acute infection in a community setting. Lancet 2006; 367: 1075-1079.

[5] Schmidt M, Horvath-Puho E, Thomsen RW, Smeeth L and Sørensen HT. Acute infections and venous thromboembolism. J Intern Med 2012; 271: 608-618.

[6] Wang L, Gong Z, Liang A, Xie Y, Liu SL, Yu Z, Wang L and Wang Y. Compromised T-cell immunity and virus-like structure in a patient with pulmonary hypertension. Am J Respir Crit Care Med 2010; 182: 434-435.

[7] Wang L, Zhang X, Duan Q, Lv W, Gong Z, Xie Y, Liang A and Wang Y. Rod-like bacteria and recurrent venous thromboembolism. Am J Respir Crit Care Med 2012; 186: 696.

[8] Xiang-Hua Y, Le-Min W, Ai-Bin L, Zhu G, Riquan L, Xu-You Z, Wei-Wei R and Ye-Nan W. Severe acute respiratory syndrome and venous thromboembolism in multiple organs. Am J Respir Crit Care Med 2010; 182: 436-437.

[9] Wang L, Song H, Gong Z, Duan Q and Liang A. Acute pulmonary embolism and dysfunction of CD3+ CD8+ T cell immunity. Am J Respir Crit Care Med 2011; 184: 1315.

[10] Lv W, Wang L, Duan Q, Gong Z, Yang F, Song H and Song Y. Characteristics of the complement system gene expression deficiency in patients with symptomatic pulmonary embolism. Thromb Res 2013; 132: e54-7.

[11] Qaseem A, Snow V, Barry P, Hornbake ER, Rodnick JE, Tobolic T, Ireland B, Segal JB, Bass EB, Weiss KB, Green L and Owens DK. Current diagnosis of venous thromboembolism in primary care: a clinical practice guideline from the American Academy of Family Physicians and the American College of Physicians. Ann Intern Med 2007; 146: 454-458.

[12] McLaughlin VV, Archer SL, Badesch DB, Barst RJ, Farber HW, Lindner JR, Mathier MA, McGoon MD, Park MH, Rosenson RS, Rubin LJ, Tapson VF and Varga J. ACCF/AHA 2009 expert consensus document on pulmonary hypertension: a report of the American College of Cardiology Foundation Task Force on Expert Consensus Documents and the American Heart Association: developed in collaboration with the American College of Chest Physicians, American Thoracic Society, Inc., and the Pulmonary Hypertension Association. Circulation 2009; 119: 2250-2294.

[13] Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, Yokoyama WM and Ugolini S. Innate or adaptive immunity? The example of natural killer cells. Science 2011; 331: 44-49.

[14] Ohrui T, Takahashi H, Ebihara S, Matsui T, Nakayama K and Sasaki H. Influenza A virus infection and pulmonary microthromboembolism. Tohoku J Exp Med 2000; 192: 81-86.

[15] Görek A, Akçay S, Ibiş OA, Atar I and Eyüboğlu FO. Herpes simplex virus infection, massive pulmonary thromboembolism, and right atrial thrombi in a single patient: case report. Heart Lung 2007; 36: 148-153.

[16] Mashav N, Saar N, Chundadze T, Steinvil A and Justo D. Epstein-Barr virus-associated venous thromboembolism: a case report and review of the literature. Thromb Res 2008; 122: 570-571.

[17] Justo D, Finn T, Atzmony L, Guy N and Steinvil A. Thrombosis associated with acute cytomegalovirus infection: a meta-analysis. Eur J Intern Med 2011; 22: 195-199.

[18] Bibas M, Biava G and Antinori A. HIV-Associated Venous Thromboembolism. Mediterr J Hematol Infect Dis 2011; 3: e2011030.

[19] Zhu T, Carcaillon L, Martinez I, Cambou JP, Kyndt X, Guillot K, Vergnes MC, Scarabin PY and Emmerich J. Association of influenza vac-

cination with reduced risk of venous thromboembolism. Thromb Haemost 2009; 102: 1259-1264.

[20] Nakata S, Imagawa A, Miyata Y, Yoshikawa A, Kozawa J, Okita K, Funahashi T, Nakamura S, Matsubara K, Iwahashi H and Shimomura I. Low gene expression levels of activating receptors of natural killer cells (NKG2E and CD94) in patients with fulminant type 1 diabetes. Immunology Lett 2013; 156: 149-155.