Genomic characteristics of adhesion molecules in patients with symptomatic pulmonary embolism

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Abstract. The aim of this study was to identify the differences in the expression of cell adhesion molecule-related mRNAs between symptomatic pulmonary embolism (PE) and a control group, and to investigate the interactions among activated leukocytes, platelets and endothelial cells. Whole human gene chip was applied to detect the expression of cell adhesion molecule-related mRNAs in symptomatic PE and in the control group, and statistical analysis was performed. In patients with PE, the expression of the majority of integrin mRNAs located on leukocytes and platelets was significantly upregulated. The expression of mRNAs related to L-selectin and P-selectin glycoprotein ligand was significantly upregulated, while the expression of mRNA related to E-selectin was significantly downregulated. The expression of mRNAs related to classic cadherins and protocadherins was downregulated, and the expression of mRNAs related to vascular endothelial cadherin was significantly downregulated; the expression of mRNAs related to the immunoglobulin superfamily had no obvious difference between the 2 groups. In conclusion, we demonstrated that, in symptomatic PE patients, the adhesion of leukocytes and platelets was enhanced; the activation of endothelial cells was obviously weakened; the adherens junctions among endothelial cells were weakened, with the endothelium becoming more permeable.

Introduction

Pulmonary embolism (PE) consists of acute pulmonary thromboembolism (APE) and chronic thromboembolic pulmonary hypertension (CTEPH). PE together with deep venous thrombosis (DVT) is termed venous thromboembolism (VTE). PE has become an international health care problem due to its high morbidity, misdiagnosis rate and mortality (1)

More than a century ago, Vichrow proposed a theory known as Virchow's triad, stating that thrombosis results from a combination of hemodynamic changes (stasis, turbulence), hypercoagulability and vessel wall injury (2). This theory forms the basis of the pathogenesis of artery thrombosis and VTE. The American Collage of Chest Physicians (ACCP) proposed a risk stratification for VTE and recommended different methods of prevention for patients at different risk levels (3). As a matter of fact, some patients at the same risk level and in a similar external environment, develop VTE after surgery, but the majority do not. It is still not clear as to why the incidence of VTE increases with age and why patients with a malignant disease are prone to VTE. It is also still not clear as to why artery thrombus is a 'white clot' and VTE a 'red clot'. Although some patients with DVT/PE received warfarin continuously, and the D-dimer level was regulated within a good range, their pulmonary artery pressure still increased gradually and they eventually developed CTEPH. ACCP summarized the risk factors for VTE as: trauma, surgery, increasing age, malignancy, pregnancy, heart failure, immobility and estrogen-containing oral contraception (3,4). Although guidelines for the prevention, diagnosis and treatment of VTE have been published 8 times from 1995 to 2008 and have been updated continuously, the clinical confusion associated with VTE remains. In 2008, Shackford reviewed the records of 37,615 patients with symptomatic VTE on surgery services over the 10-year period since the initial publication of the ACCP guidelines, of which 84% were either in partial or complete compliance with the guidelines. The incidence of VTE, however, increased gradually over the years of the study, instead of decreasing (5). The possible reason for theory and clinical practice being separated is that the etiology and pathogenesis of VTE are not yet clear.

In 2006, Smeeth reported that VTE was associated with infection, especially in the first 2 weeks following infection (6). In 2010 it was also reported that VTE was found in multiple organs in a patient, who died of severe acute respiratory syndrome (SARS), indicating that a viral infection caused systemic VTE (7). In a previous study, we identified a virus-like structure in T cells in a patient with CTEPH, which proved that T cells were infiltrated by a virus (8). In 2011, it was also reported that $CD3^+$ and $CD8^+$ T cell-mediated immune deficiency or compromise occurred in patients with CTEPH (9)

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and acute PE (10). Our research focused on the correlation between VTE and infection, inflammation, and immunity.

There are numerous leukocytes aggregating at the site of a thrombus in VTE. The adhesion among leukocytes, endothelial cells and platelets occurs throughout the process of VTE (11,12). Cell adhesion molecules (CAMs) are a type of glycoprotein expressed on the cell surface mediating cell-cell and cell-matrix interactions, which are the basis of cell adhesion. CAMs participate in a series of physiological and pathological processes, including signal transduction and activation, morphogenic movements, cellular migrations, cell growth and differentiation, as well as inflammation, thrombosis, wound healing and metastasis. Hundreds of CAMs have been identified in humans and are divided into 4 families: integrins, selectins and the immunoglobulin and cadherin superfamily. In this research, a whole human gene expression chip was applied to detect the differences of CAM-related mRNA expression in patients with PE and in a control group. The correlations among CAMs in activated leukocytes, platelets and endothelial cells during the process of PE were investigated.

Materials and methods

Patient information. The 20 patients enrolled in the PE group were those who were admitted in hospital during the year 2007, and included 11 males and 9 females, with an average age of 70±14 years (44-89 years old). All patients were diagnosed with PE on the basis of at least 2 of the following criteria: i) selective pulmonary arteriography showing a filling defect or blockage; ii) pulmonary ventilation perfusion scanning exhibiting single or multiple blood flow perfusion defects with normal or abnormal ventilation and mismatched ratio of ventilation/perfusion; iii) other clinical characteristics, including a typical manifestation of PE, arterial blood gas analysis, D-dimer test, ultrasound cardiogram (UCG) and chest computerized tomography (CT) supported the diagnosis and excluded other cardiac and pulmonary disorders. Another 20 patients with ischemic heart disease admitted during the same period, without PE, DVT and other congenital bleeding and thrombosis diseases with comparative clinical presentation (11 males, 9 females; 44-91 years of age with a mean age of 72±14) were enrolled in the control group. The study protocol was approved by the local ethics committee and an informed consent was obtained from all the patients in accordance with the declaration of Helsinki.

Total RNA isolation. A total of 5 ml of peripheral blood samples anti-coagulated with EDTA were drawn from patients suspected of having PE and from those without PE, immediately after being admitted to the hospital. Mononuclear cells were obtained through density gradient centrifugation with Ficoll solution and the remaining red blood cells were destroyed by erythrocyte lysis buffer (Qiagen, Hilden, Germany). Total mononuclear cell RNA was extracted with TRIzol (Invitrogen, Carlsbad, USA) and purified with Qiagen RNeasy column (Qiagen), according to the manufacturer's instructions. The isolated total RNA was tested and quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technology, Cambrige, UK).

Gene expression clip. Agilent G4112A Whole Human Genome Oligo Microarrays were purchased from Agilent (USA). A microarray is composed of 44,290 spots including 41,675 genes or transcripts, 314 negative control spots, 1,924 positive control spots and 359 blank spots. The functions of more than 70% of the genes in the microarray are already known. All patients were subjected to clip analysis.

Target preparation and microarray hybridization. The RNA samples of patients with confirmed diagnosis of PE and controls were labeled using the indirect labeling method. Briefly, 1 μg of total RNA was reverse transcribed. Second strand cDNA was then produced and purified followed by *in vitro* transcription (IVT) with T7 RNA Polymerase. During IVT, the modified nucleotide, 5-(3-aminoallyl)-UTP (aaUTP) was incorporated into the cDNA. Subsequently, the fluorescent Cy3 was chemically coupled with the aaUTP which contains a reactive primary amino group on the C5 position of uracil. The dye incorporation rate was assessed with a Nanodrop ND-1000 spectrophotometer and was found to be between 1.2-1.4 pmol/μl. Hybridization was carried out using the Agilent Oligonucleotide Microarray *in situ* Hybridization Plus kit (p/n 5,184-3,568), according to the manufacturer's instructions. Briefly, 750 ng of Cy3-labeled sample cDNA was subjected to fragmentation (30 min at 60˚C) and then hybridization on 44K Human Whole-Genome 60-mer oligo-chips (G4112F, Agilent Technologies) in a rotary oven (10 rpm, 60˚C, 17 h). Slides were disassembled and washed in solutions I and II, according to the manufacturer's instructions.

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

Statistical analysis. The Agilent Feature Extraction software was used to collect the original data from the microarray, followed by an analysis with a robust multichip average (RMA). The gene intensity data between the PE and control group were compared with a random variance model-corrected t-test. Differentially expressed genes were identified from whole genomes. A p-value <0.05 was considered to indicate a statistically significant difference.

Results

Integrin mRNA expression

Leukocyte-related integrin mRNA expression. Among the 13 mRNAs, 7 were upregulated (of which 5 significantly) in the PE group, compared with the controls ($p<0.05$); 6 were downregulated (of which 1 significantly) in the PE group ($p<0.05$) (Fig. 1A).

Platelet-related integrin mRNA expression. Of the 7 mRNAs, ITGA2, ITGA5, ITGA6 and ITGB1 were also leukocyte-related integrin mRNAs. Five mRNAs were upregulated (of which 4 significantly) ($p<0.05$); 2 mRNAs were

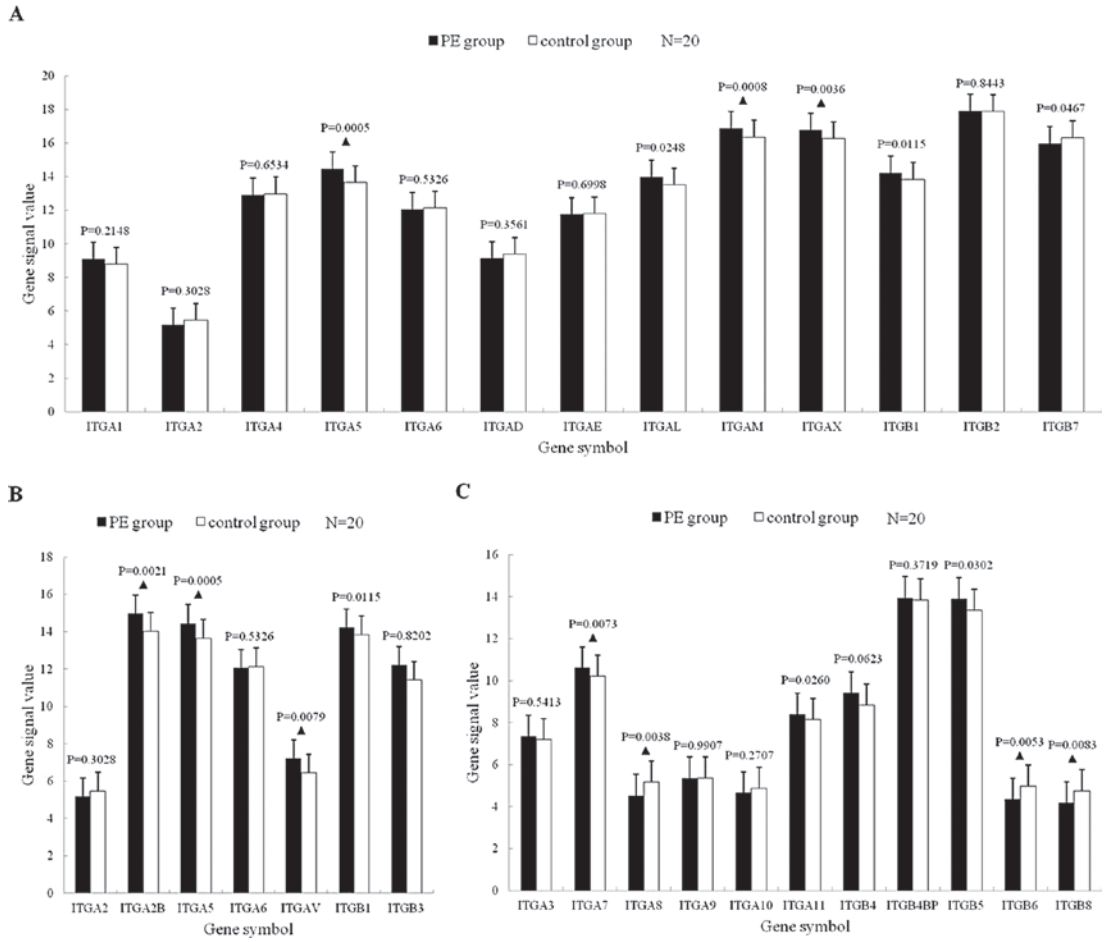

Figure 1. Expression of integrin mRNAs. (A) leukocyte-related integrin mRNA expression; (B) platelet-related integrin mRNA expression; (C) other integrin mRNA expression. *p<0.05; *p<0.01.

downregulated, but with no statistically significant difference (p>0.05) (Fig. 1B).

Other integrin mRNA expression. Among the 11 mRNAs, 6 were upregulated (of which 3 significantly) in the PE group (p<0.05) and 5 were downregulated (of which 3 significantly) (p<0.01) (Fig. 1C).

Selectin mRNA expression. Among the 4 mRNAs, SELL and SELPLG mRNA expressions were significantly upregulated (p<0.01), while the SELE mRNA expression was significantly downregulated (p<0.01) (Fig. 2A).

Immunoglobulin superfamily mRNA expression. There were no statistically significant differences in the 5 mRNAs between the PE and control group (p>0.05) (Fig. 2B).

Cadherin superfamily mRNA expression

Classic cadherin mRNA expression. Among the 13 mRNAs, 10 were downregulated (of which 7 significantly) in the PE group (p<0.05) and 3 were upregulated (of which 1 significantly) (p<0.05) (Fig. 2C).

Protocadherin mRNA expression. The 34 mRNAs consisted of 13 non-clustered and 21 clustered protocadherins. A total of 19 mRNAs were significantly downregulated (p<0.05), of which 9 more significantly (p<0.01); 3 mRNAs were upregulated significantly (p<0.05), of which 2 more significantly (p<0.01) (Fig. 3).

Flamingo cadherin mRNA expression. The 3 mRNAs were all upregulated, without a statistically significant difference (p>0.05) (Fig. 2D).

Discussion

Expression profile of integrin mRNAs

Expression profile of leukocyte-related integrin mRNAs. Among the 13 mRNAs, 5 (38.46%) were upregulated with a statistically significant difference (p<0.05). In these 5 mRNAs, the ITGAL, ITGAM and ITGAX transcripts (subunit αL, αM and αX) bind to subunit β2 to generate β2 integrins, and are expressed on leukocytes specifically; the ITGA5 and ITGB1 transcripts are subunits of α5 and β1. β1 binds to α1-α11 and αV to generate β1 integrins (13).

The 3 α subunits of β2 integrins were all significantly upregulated with a statistically significant difference, indicating that connections between β2 integrins and their ligands were enhanced during the interaction between leukocytes and vascular endothelial cells, a fact that suggests that the adhesion of β2 integrins was enhanced. In the β1 integrins, only α5 and β1 were significantly upregulated with a statistically significant difference. α5β1 was also expressed in platelets,

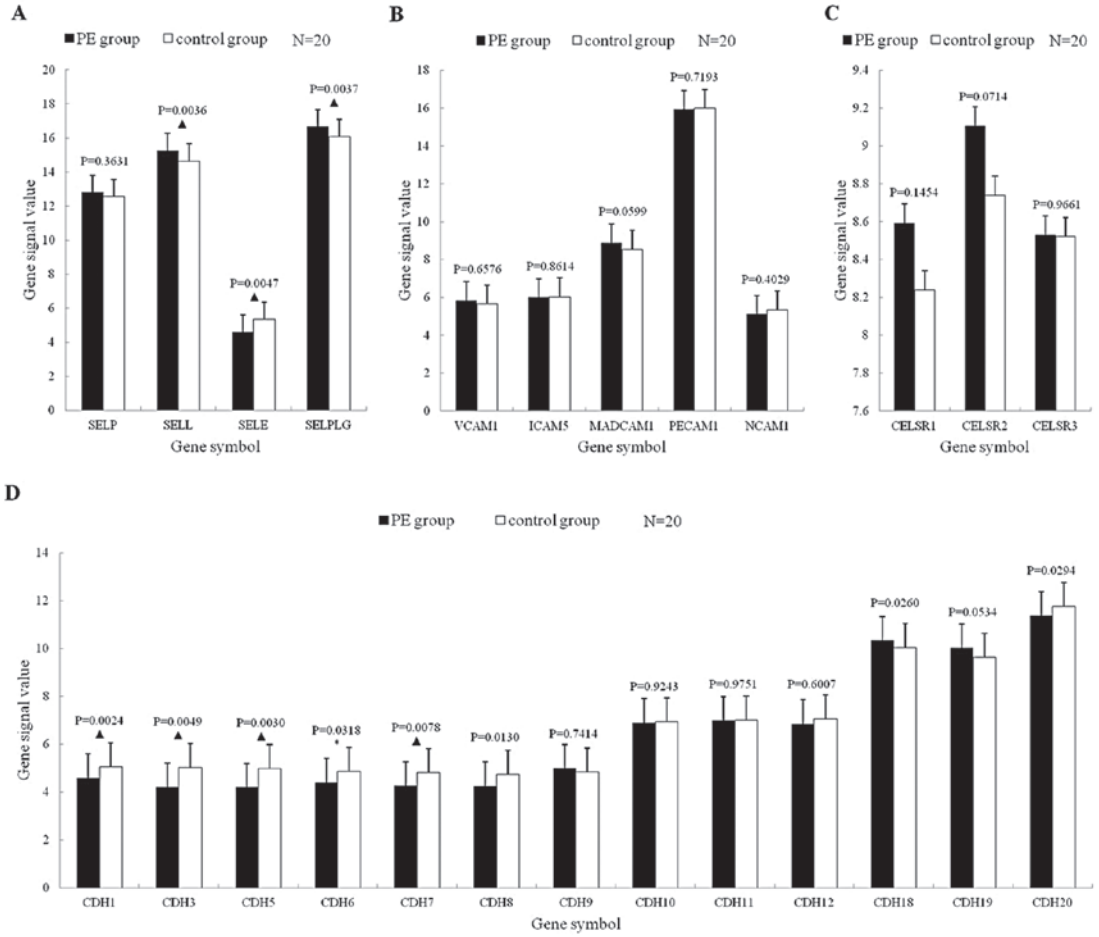

Figure 2. Expression of selectin, immunoglobulin superfamily, flamingo cadherin and classic cadherin mRNAs. (A) Selectin mRNA expression; (B) immunoglobulin superfamily mRNA expression; (C) flamingo cadherin mRNA expression; (D) classic cadherin mRNA expression. *p<0.05; ▲p<0.01.

indicating that the upregulation of the subunits α5 and β1 occurred in leukocytes and platelets at the same time.

Thus, we considered that the signal transduction of β1 integrins was enhanced and leukocytes actively reinforced adhesion to the vascular endothelial cells in PE patients. The αM and αX mRNAs were significantly upregulated. αMβ2 was expressed in monocytes, macrophages and neutrophils, while αXβ2 was expressed in monocytes, NK cells, neutrophils and some lymphocytes. Caimi *et al* reported that neutrophils were activated abnormally in VTE (14,15). The genomics results were consistent with the cytology results.

Expression profile of platelet-related integrin mRNAs. There are 5 integrins expressed in platelets: αIIβ3 (GPIIb/IIIa), αVβ3, α2β1, α5β1 and α6β1. In our research, the 2 subunits of GPIIb/IIIa were both significantly upregulated. Most GPIIb/IIIa copies are present on resting platelets, while another small part is hidden in platelet α-granules. The GPIIb/IIIa copies on α-granules become externalized on platelet secretion to increase their surface expression by 25 to 50%, and expose their binding sites for fibrinogens to become activated (16).

Expression profile of other integrin mRNAs. In these 11 mRNAs, the ITGA11 and ITGA7 transcripts (α11 and α7) bind to subunit β1 to generate α11β1 and α7β1; the ITGB5 (β5) transcript binds to subunit αV to generate αVβ5. The ligands of α11β1, α7β1 and αVβ5 are collagen, laminin and fibronectin, respectively (17), indicating that the signal transduction and the binding of integrins to collagen, laminin and fibronectin are enhanced in PE patients. In the 3 mRNA transcripts which were significantly downregulated, the subunit α8 binds to β1 to generate α8β1; β6 and β8 bind to αV to generate αVβ6 and αVβ8. Major ligands of α8β1, αVβ6 and αVβ8 are all fibronectin.

In the PE group, most upregulated mRNAs of the leukocyte-related integrin transcribed into β1 and β2 integrins; 60% of platelet-related integrin mRNAs were upregulated which transcribed into β1 and β3 integrins, indicating that integrins expressed in leukocytes and platelets play an important role in the PE process, while the upregulation of β1 integrin mRNAs was related to both leukocytes and platelets. Most transcripts of other integrin mRNAs which were abnormally expressed bound to fibronectin, indicating that fibronectin plays a role in the onset of VTE.

Expression profile of selectin mRNAs. P-selectin is stored in the a-granules of platelets and the Weibel-Palade bodies of endothelial cells (18); E-selectin is expressed on the surface of activated leukocytes specifically (19); L-selectin is expressed on the surface of most leukocytes (20); P-selectin glycoprotein ligand (PSGL-1) is expressed mainly on the surface of leukocytes and platelets (21). PSGL-1 is a receptor of P-selectin with

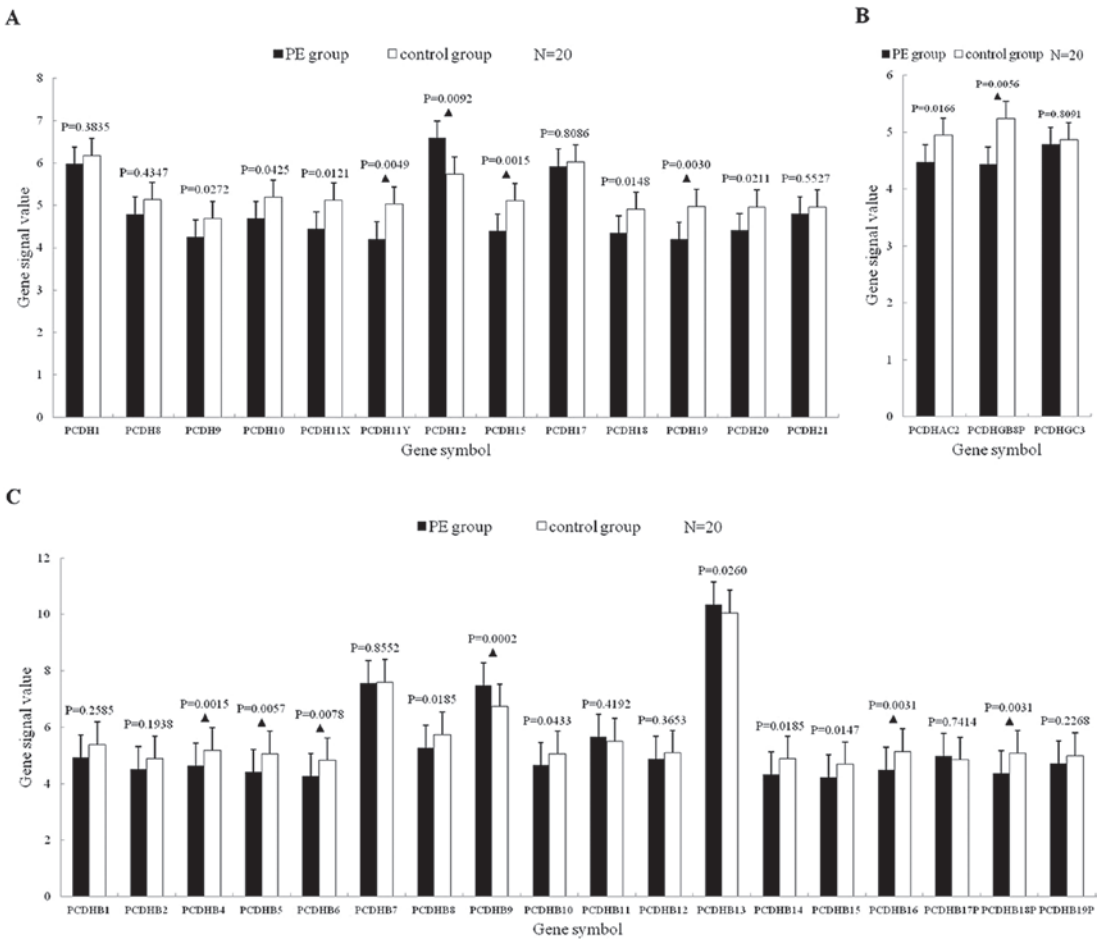

Figure 3. Expression of protocadherin mRNAs. (A) Non-clustered protocadherin mRNA expression; (B) clustered protocadherin α and γ mRNA expression; (C) clustered protocadherin β mRNA expression. *p<0.05; *p<0.01.

high affinity that can bind both to L-selectin and E-selectin to mediate interactions among leukocytes, platelets and endothelial cells. In our research, L-selectin and PSGL-1 mRNAs were significantly upregulated, indicating that leukocytes were activated in PE patients; E-selectin mRNA was significantly downregulated, indicating that the activation of endothelial cells was obviously weakened, while no statistically significant difference in P-selectin between the PE and the control group was observed.

Expression profile of immunoglobulin superfamily mRNAs. The expression trends of VCAM-1 mRNA and PECAM-1 mRNA in PE patients were similar to those in artery thrombosis (22); no statistically significant difference in the expression of MadCAM-1, ICAM-1, NCAM-1 between the PE and the control group was observed, indicating that the immunoglobulin superfamily did not play an important role in PE.

Expression profile of cadherin superfamily mRNAs

Expression profile of classic cadherin mRNAs. Among the 13 mRNAs, 7 (53.84%) mRNAs were downregulated statistically, of which 4 (30.77%) significantly. Vascular endothelial cell cadherin (VE-cadherin) expressed on the surface of endothelial cells is the major endothelial adhesion molecule of the adherens junction, and negatively regulates the transendothelial migration of leukocytes (23). During inflammation, leukocytes weaken the negative regulatory effect of VE-cadherin by VE-cadherin phosphorylation (24), as well as the binding of VE-cadherin to endothial cells (25,26) and cleave VE-cadherins (27) to promote their transendothelial migration, enhance leukocyte infiltration and cause inflammation. In this research, VE-cadherin mRNA expression was significantly downregulated, indicating that the adherens junction between vascular endothelial cells was obviously weakened and that the permeability of the endothelium was increased in PE patients.

Expression profile of protocadherin mRNAs. A total of 19 (55.88%) mRNAs were significantly downregulated among the 34 protocadherin mRNAs (p<0.05), of which 9 (26.47) more significantly (p<0.01), indicating that protocadherins were downregulated as a whole in VTE. The result was similar to classic cadherins. Similar to VE-cadhrein, VE-cadherin 2 was also expressed on the adherens junction among endothelial cells; however, in contrast to VE-cadherin, its mRNA expression was upregulated in PE patients.

Expression profile of flamingo cadherin mRNAs. Flamingo cadherins are a type of 7-pass transmembrane protein (28). In this research, the 3 mRNAs of this sub-family were all upregulated in PE patients, but with no statistically significant difference.

During the process of PE, leukocytes were significantly activated with enhanced adhesion, the adhesion of platelets was enhanced and the activation of vascular endothelial cells and the adherens junction between endothelial cells were weakened, with the endothelium becoming more permeable. During the process of PE, leukocytes increased their ability to adhere and were assisted by platelet adhesion. The vascular endothelial cell adhesion, however, may be passive. The obvious weakness of the adherens junction among endothelial cells provided the conditions for the migration of inflammatory cells.

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