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Original Article

Characterization of immune cells and perforin mutations in familiar venous thromboembolism

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Abstract: Aim: This study was to carry out exome sequencing in a Han Chinese family with venous thromboembolism. Methods: Three venous thromboembolism (VTE) patients and five members from a Han Chinese family were evaluated by exome sequencing. Results: Among the 3 VTE patients, mutations of 2 genes including *PRF1* and *HTR2A* were identified and predicted to be functionally damaged to their encoded proteins. In addition, the *PRF1* mutation and the *HTR2A* mutation identified in our study were absent in 100 non-related controls, indicating that venous thromboembolism has a genetic component. The R357W mutation is located in the membrane attack complex/perforin domain of *PRF1* protein, which exists in both the perforin. The steps of killing foreign or pathological antigen cells by NK cells, CD_8^+T cells and the membrane attack complex include membrane perforation and release of the granzyme, either of which is abnormal can lead to immune dysfunction. Conclusions: The mutations of immune related genes in familial VTE might provide new understanding of the pathogenesis of familial venous thromboembolism.

Keywords: Family, genes, mutation, perforin, venous thromboembolism

Introduction

Venous thromboembolism (VTE) is a common medical condition which includes deep vein thrombosis (DVT) and pulmonary embolism (PE). PE is a major public health problem with a high incidence, mortality, misdiagnosis and missed diagnosis rate [1]. The American College of Chest Physicians (ACCP) has published guidelines for the prevention, diagnosis, and treatment of VTE which have been regularly updated from 1995 to 2012 [2]. Acquired risk factors for VTE include infection, malignancy, increasing age, surgery and trauma. In addition it has been proposed that family history might increase the risk of VTE. A 22-year study, from 1987 to 2009, demonstrated that family history was an important risk factor for VTE, and supported a strong genetic component to VTE [3]. It has been reported so far that inherited risk factors for VTE are rare and mainly consist of loss-of-function mutations in genes encoding anticoagulant proteins and gain-of-function mutations in genes encoding procoagulant proteins [4].

We have reported that the occurrence of symptomatic VTE was associated with decreased and/or disordered immune function [5]. Transmission electron microscopy showed rod-like bacteria in the phagocytes of a patient with recurrent PE/DVT and virus-like microorganisms in the CD_3^+ and CD_8^+T cells of a patient with VTE/PAH, respectively [6, 7]. We have reported that a common immunological feature of these patients is that they had decreased number of CD_3^+ T cells and CD_8^+T cells, and increased CD_4^+/CD_8^+ ratios [8, 9]. We have also reported that venous thrombosis was found in multiple organs including the lungs, spleen, pancreas, kidneys, and adrenal glands from a patient who died of severe acute respiratory syndrome [10], indicating that virus and bacterial infections were associated with the occur-

rence of VTE. Infection is a risk factor for VTE, raising the possibility that disordered immune function may be a contributory factor by facilitating infection.

We have analyzed the role of immune cells in the formation of thrombosis in the lung vessels of mice after influenza A/H1N1 infection. To confirm previous clinical findings, an A/H1N1 influenza infection model was established to compare the pulmonary thrombosis in 6 groups of infected mice with different immune statuses (C57BL/6, BALB/c: without deficiency of immune cells; Scid: T, B cells combined deficiency; NOD/LtJ: NK cell deficiency; BALB/c-nu: T cell deficiency; NOD-Scid: T, B, NK cells combined deficiency). The results showed the virus could result in thrombosis in pulmonary small arterials, small veins and capillary vessels. In 4 groups of mice with deficiency of T cells, B cells, NK cells or combined cell deficiency, the incidence rate of thrombosis was significantly greater than that of mice without cellular immune deficiency. Several animal experiments have demonstrated that different types of immune deficiencies can lead to the occurrence of VTE, especially combined immune deficiencies.

Although it has been demonstrated by clinical and animal experiments that pathogenic microorganisms can trigger the occurrence of VTE in the presence of immune dysfunction, but the reason of immune abnormalities is unclear. In the present study, we carried out exome sequencing in a Han Chinese family with VTE, and it is reported as follows.

Materials and methods

Clinical data of this Chinese family

A Han Chinese family (12 members) with VTE was used in this study (**Figure 1A**). In the first generation, 2 members (1 with a history of DVT) died; in the second generation, 1 member with a history of DVT died and 3 members were affected; in the third and fourth generation, 5 members were healthy.

The proband (II:9), a 50-year-old man, initially presented with right leg swelling at the age of 41. Computed tomography (CT) scans revealed deep vein thrombosis in the right leg. After 1 month of treatment with warfarin, the symptoms disappeared. X years later, he presented with shortness of breath, cough, and bilateral lower extremity edema, slightly worse on the right. The value of D-dimer of the proband was 0.89 mg/L (reference range: <0.3 mg/L). White blood cell (WBC) count was 6.2×10^9/L (reference range: $3.69\text{-}9.16\times10^9$/L) with 53.7% (reference range: 50-70%) neutrophils. CT angiography of pulmonary vessels at onset showed filling defect in lower left pulmonary artery. Contrast-enhanced CT scan of lower abdomen at onset showed right superior external iliac vein and femoral vein thrombosis.

The proband's father (I:1) who had a history of lower limb DVT, died suddenly at the age of 60, and the cause of his death was unknown. The proband's mother (I:2) died of natural causes at the age of 80. The proband's older brother (II:1) who had a history of lower limb DVT, died of cerebral infarction at the age of 37. The proband's older sister (II:3) presented with right leg swelling at the age of 58. Venous ultrasonography of lower limb at onset showed right popliteal vein and small saphenous vein thrombosis, and she was diagnosed with DVT. The proband's other older sister (II:7) presented with left lower extremity edema at the age of 56. Venous ultrasonography of lower limb revealed left lower limb deep vein thrombosis.

Exon capture and next-generation sequencing

Exome sequencing was performed on 8 members of the family. Genomic DNA was extracted from peripheral blood using QIAamp DNA Blood Midi Kit and sheared by sonication. Exonic DNA was then captured using SureSelect Human All Exon 50Mb kit (Agilent Technologies). The kit contains a pool of RNA-based 120-mer capture oligomers targeting 51,646,629 bases of 213,384 consensus coding sequences and their flanking regions. The Illumina Hiseq2000 platform was applied in sequencing the exon-enriched DNA following the manufacturer's instructions.

Sequence mapping and single nucleotide variation identification

Raw sequencing reads were processed by Fastx-toolkit pipeline (http://hannonlab.cshl.edu/fastx_toolkit) to remove adapter sequences and low quality sequences. Then the reads were aligned to the human reference genome sequences, UCSC hg19 (http://hgdownload.soe.ucsc.edu/downloads.html#human) using

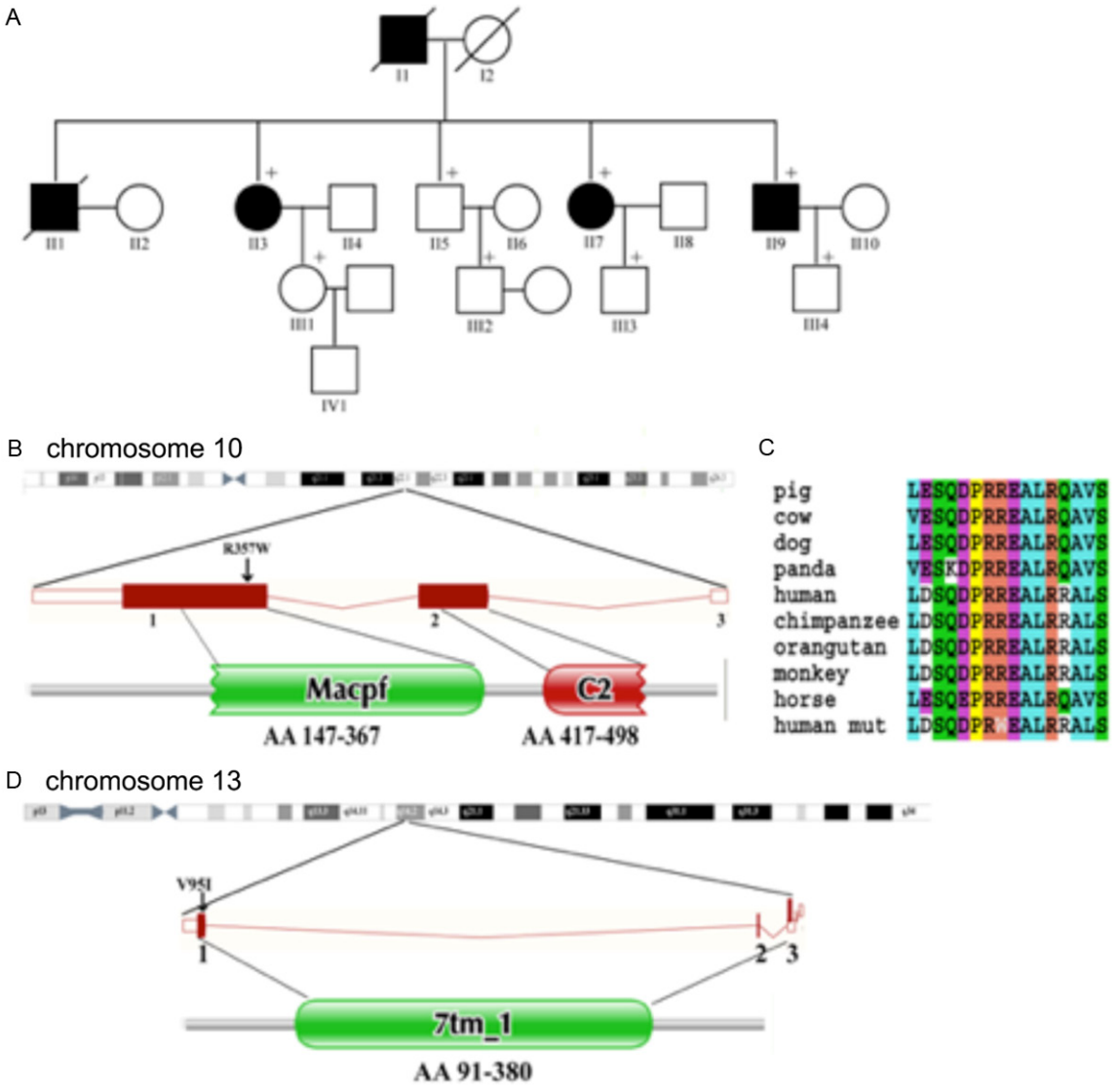

Figure 1. Pedigree and *PRF1* and *HTR2A* mutations of the VTE family. A. The genealogical tree of VTE family. + indicates family members who were examined and sequenced in this study. Filled symbols indicate affected individuals. B. The chromosomal location and genomic structure of the exons encoding the open reading frame of *PRF1*. The R357W mutation locates in the MACPF domain of protein. C. *PRF1* orthogous conservation analysis was performed using CLUSTALW at default settings. The *PRF1* R357W mutated sequence is shown in white. D. The chromosomal location of *HTR2A* and the location of its exons. The exon numbering indicates *HTR2A* is located on the reverse strand and the V95I mutation is location on exon 1.

Burrows-Wheeler Aligner (BWA) software [11]. Sequence reads which could not be aligned to the designed target regions were filtered out and duplicate reads was further removed by SAMtools software [12]. Variations including single nucleotide variation (SNV) as well as small insertion and deletion (Indel, <10 bp) were called with the SAMtools software package. The variations should meet the following criteria: mapping quality ≥40, SNV quality ≥20, Indel quality ≥50 and ≥5 reads covered. Any two or more SNVs located in a 5-bp window or SNVs overlapping with an Indel were discarded. The SNVs identified were further filtered by dbSNP 137, HapMap database (ftp://ftp.ensembl.org/pub/release-62/variation/homo_sapiens/) and 1000 Genomes databases and to remove known SNVs and variants remained were considered to be 'novel' SNVs. Gene transcript annotation databases (http://snp-nexus.org/index.html) were used for transcript identification and for determining amino acid chang-

Table 1. List of mutations in 3 genes predicted to be damaging shared by 3 affected individuals

Chr	Site	Ref	Mut	Gene	II3[a]	II5[b]	II7[a]	II9[a]	III1[b]	III2[b]	III3[b]	III4[b]
chr10	72358408	G	A	*PRF1*	Y[c]	N	Y	Y	N	N	N	N
chr13	47469759	C	T	*HTR2A*	Y	N	Y	Y	N	N	N	N
chr11	17482154	G	A	*ABCC8*	Y	N	Y	Y	Y	N	N	N

Footnotes: a, Patients in the VTE family; b, Controls in the VTE family; c, "Y" means the individual carries the amino acid alteration, and "N" means does not.

Table 2. Expression of differentiation antigens on immune cells and complements in 8 members of the VTE family

	II:3	II:5	II:7	II:9	III:1	III:2	III:3	III:4
Age at Examination (years)	62	59	56	50	35	30	26	21
CD3 (60-85 %)	74.7	52[a]	76.3	78.6	48.7[a]	56.7[a]	69.3	80.1
CD4 (24.5-48.8 %)	44.1	30.5	51.8[b]	45.3	27.3	23.4[a]	35.7	30.7
CD8 (18.5-42.1 %)	23.4	18.6	20.8	31.8	18.0[a]	26.1	29.7	35.3
CD4/CD8 (1.02-1.94 %)	1.88	1.6	2.49[b]	1.42	1.52	0.9[a]	1.2	0.87[a]
NKCD16+CD56+ (8.6~21.1%)	5.32[a]	23.2[b]	7.13[a]	7.54[a]	42.8[b]	25.9[b]	15.7	15.0
CD19 (8~20 %)	11.4	16.9	6.35[a]	10.0	8.1	1.9	8.3	6.16[a]
CH50 (23-46 U/ml)	48[b]	-	42	49.6[b]	38	-	-	50↑
C3(0.9-1.8 g/L)	0.99	1.19	0.92	1.2	1.03	1.52	1.05	1.69
C4(0.1-0.4 g/L)	0.26	0.26	0.23	0.26	0.18	0.31	0.21	0.32

Footnotes: [a]indicates the level was less than the lower limit of the normal range; [b]indicates the level was higher than the upper limit of the normal range.

es of these 'novel' SNVs [13]. Amino acid changes were annotated according to the largest transcript of genes. PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/) was applied to assess the functional impact of non-synonymous SNVs [14].

Examining PRF1, HTR2A and ABCC8 mutations in control individuals

The mutation of *PRF1*, *HTR2A* and *ABCC8* were further examined in 100 ethnically matched non-related controls. Primers for both PCR amplification and Sanger sequencing were as following: CCGCTGAGCCATGGCACACA (Forward), and CGGAAGTGGTTGGCGGCCAT (Reverse) for PRF1, TGGACACGGGCATGACAAGGA (Forward), and AGCTCAACTACGAACTCCCTAATGCA (Reverse) for HTR2A and CCCCTGGGGCTGCCTACCTT (Forward), and CCCCAGACAACAGGAGCTAGG (Reverse) for *ABCC8*, respectively.

Examination of differentiation antigens on immune cells

2 ml of fasting venous blood was obtained from 8 members in the morning, and then the sample was added into the ET tube. The differentiation antigens on the immune cells including CD_3^+, CD_4^+, CD_8^+, CD19 and $CD_{16}^+{}_{56}^+$ in 8 members of the VTE family (II:3, II:5, II:7, II:9, III:1, III:2, III:3 and III:4) were examined by BECKMANCOULTER EPICS XL-II flow cytometer.

Detection of complement components

Complement factors C3 and C4 levels were detected by nephelometry (BN II; Siemens, Germany). Total hemolytic complement (CH50) activity was measured by liposome immunoassay using an automated biochemistry analyzer (Beckman DxC-800). This study has been approved by the Ethics Committee of Tongji University, and informed consent form was also obtained.

Results

Exome sequencing

Exome sequencing was performed on 3 affected individuals (II:3, II:7 and II:9) and 5 unaffected members (II:5, III:1, III:2, III:3 and III:4) in the VTE family, respectively (**Figure 1A**). An average of 5.5 Gb (110-folds of the target region) raw

sequencing data was generated per individual, in which approximately 83% of the reads could be successfully mapped to the UCSC hg19 reference genome using the BWA short read aligner. After removing duplicated reads, approximately 3 Gb sequencing data per sample was obtained which could be mapped to the targeted bases with a mean coverage of 55-fold. More than 90% of the targeted bases were sufficiently covered. A total of ~30,000 single nucleotide variants (SNVs) were detected in each sample, among which 822 to 1,355 novel SNVs were not present in dbSNP 137, 1000 Genomes or HapMap databases. Only 15 SNVs were shared by 3 affected patients (II:3, II:7 and II:9), but not by all of the other unaffected members, among which mutations of *PRF1*, *HTR2A*, and *ABCC8* were predicted to be functionally damaged to their encoded proteins based on PolyPhen2 mutation annotation databases (**Table 1**). Further Sanger sequencing identified *ABCC8* R298C mutation in one of 100 ethnically matched control individuals, suggesting it is unlikely to be a pathogenic mutation of VTE.

Mutations of 2 genes predicted to be functionally damaged

PRF1 R357W mutation

PRF1 gene (perforin 1, pore forming protein) consists of 3 exons and encodes a protein of 555 amino acids. The protein is a key effector molecule for T-cell- and natural killer-cell-mediated cytolysis, and capable of lysing a variety of target cells non-specifically. **Figure 1B** showed that the R357W mutation located in the MACPF domain of *PRF1* protein, which also existed in the membrane attack complex proteins of complement (C6, C7, C8α, C8β and C9) [15]. To study the evolutionary conservation of the R357 residue, a CLUSTALW analysis was performed on orthogous of the *PRF1* of different mammal species. **Figure 1C** shows the results of the orthologous conservation analyses; the conclusion drawn is that the R357 residue is conserved in evolution and has never been observed to be mutated.

HTR2A V95I mutation

The *HTR2A* V95I is another mutation not reported in VTE. It consists of 3 exons encoding 471 amino acids, and associated with susceptibility to schizophrenia and obsessive-compulsive disorder [16]. **Figure 1D** shows V95I mutation in the 7tm_1 domain of *HTR2A*.

Expression of differentiation antigens on immune cells and complements were detected in 8 members of the VTE family (**Table 2**), and it showed that the number of NK cells was decreased, the levels of CD8, C3 and C4 were in the normal range, and the level of CH50 was significantly increased in these members.

Discussion

Exome sequencing method has been used to find a causal gene underlying rare Mendelian disorders [17]. In the present study, we examined the exome sequence of 8 members from a four-generation Han Chinese family with VTE. Among 3 affected individuals, mutations of *PRF1* gene located in chromosome 10 and *HTR2A* gene located in chromosome 13 were predicted to result in functional derangement of their encoded proteins. The R357W mutation of *PRF1* gene and the V95I mutation of *HTR2A* were detected in the 3 affected members but not in another 5 normal members of the family (II:5, III:1, III:2, III:3 and III:4) or in 100 normal controls, indicating that VTE has a genetic component.

Smeeth reported that the risk of VTE significantly increased after acute infection, and infection might be the trigger of VTE [18]. However, it is important to note that VTE only occurs as a complication in a minority of patients with infection. This raises the question of what determines whether any given infection will result in VTE.

The R357W mutation was identified in MACPF (MAC component/perforin) domain of *PRF1* protein. MACPF domain exists in both the perforin secreted by cytotoxic T lymphocytes and NK cells, and the MAC of complement (C6-C9) [19-21]. The R357W mutation of *PRF1* indicates that the structure of NK cells, CD_8^+T cells and MAC may be abnormal, which may cause impaired capability of killing infected or tumor cells. We have reported that the mRNA expression of granzyme secreted by T cells was significantly down regulated in symptomatic VTE patients, as compared to the control group [22], which suggests that the capability of killing infected or tumor cells is impaired in CD_8^+T

cells. The steps of killing foreign /or pathological antigen cells by NK cells, CD_8^+T cells and the membrane attack complex include membrane perforation and release of the granzyme, either of which is abnormal can lead to immune dysfunction. The R357W mutation identified in this report is consistent with the result that the mRNA expression of granzyme was significantly down-regulated in symptomatic VTE patients. These results are a manifestation of a decrease in different aspects of killing foreign/or pathological antigen cells, leading to decreased immune function.

The *HTR2A* which encodes one of the receptors for 5-HT, consists of 3 exons encoding 471 amino acids. Approximately 90% of the human body's 5-HT is produced and distributed in enterochromaffin cells [23]. When 5-HT is released into the blood flow, it is rapidly taken up by platelets which store it within their dense granules [24], which constitute about 8% of the total 5-HT. It has been reported that serotonin 5-HT2A receptor blockers can increase risk of VTE and the underlying mechanism is unknown [25]. In this study, the V95I mutation of *HTR2A* may lead to changes in the encoded protein. Therefore, we speculate that when serotonin cannot combine with its receptors effectively, effects are similar to the effects of serotonin 5-HT2A receptor blockers in VTE may be produced, leading to thrombus formation.

In addition to the exone analysis we also examined the possibility that there may be other causes underlying the genetic predisposition to VTE in this family. However no known VTE-related factors such as Factor V Leiden mutation, prothrombin mutation, and protein S, protein C antithrombin deficiency, Lupus anticoagulant or cardiolipin antibodies were found to be increased in members of this family.

In this study, 8 members of the Chinese family with VTE were evaluated by exome sequencing. An 18-month follow-up study of the 8 members found that no VTE events occurred in the 5 unaffected members, and no recurrent VTE occurred in the 3 affected members who have been taking warfarin. The present study suggests that VTE patients with immunodeficiency under control of heredity should receive lifelong anticoagulation for preventing VTE recurrence.

In this report, the mutations of immune related genes in familial VTE might provide new understanding of the pathogenesis of familial VTE.

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Disclosure of conflict of interest

None.

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