Proteinase 3 expression on the neutrophils of patients with paroxysmal nocturnal hemoglobinuria

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Abstract. Proteinase 3 (PR3) is released from neutrophils and regulates platelet activity, which is associated with cluster of differentiation (CD)177 antigen (NB1), a glycosylphosphatidylinositol-linked protein. In the present study, the effect of PR3 on thrombosis in paroxysmal nocturnal hemoglobinuria (PNH) and PNH-aplastic anemia (AA) syndrome was explored. The expression of PR3 and NB1 on $CD59^-$ neutrophils was detected by flow cytometry, immunofluorescence (IF), reverse transcription-quantitative polymerase chain reaction analysis and western blotting. Serum levels of PR3, proteinase-activated receptor 1 (PAR1) and D-Dimer were measured using ELISAs. The expression of PR3 and NB1 on the plasma membrane of $CD59^-$ neutrophils in patients with PNH/PNH-AA was significantly lower compared with their expression on $CD59^+$ neutrophils in patients and controls (P=0.001). However, no correlation between PR3 and NB1 expression was identified. IF staining further demonstrated partially positive PR3 expression on $CD59^-$ neutrophils. The serum level of PR3 in patients was identified to be significantly decreased compared with healthy controls (P<0.0001), and significantly negatively correlated with PAR1 (r=-0.456; P=0.043) and D-Dimer (r=-0.503; P=0.028) levels. The mRNA and protein levels of PR3 on PNH clones did not change significantly compared with the control group. In conclusion, PR3 expression on the plasma membrane of neutrophils and in the serum of patients with PNH/PNH-AA decreased, which may result in increased PAR1 expression and increased clotting. The present study provides the basis for further study on platelets in PNH.

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Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare acquired clonal disorder caused by a somatic mutation in the phosphatidylinositol N-acetylglucosaminyltransferase subunit A gene of multipotent hematopoietic stem cells (1). This leads to defects in the biosynthesis of glycosylphosphatidylinositol (GPI) and GPI-linked proteins, including complement decay-accelerating factor and the cluster of differentiation (CD)59, which are particularly sensitive to complement regulation (1-3). As a consequence, the absence of GPI-linked proteins induces intravascular hemolysis, bone marrow failure and life-threatening venous thrombosis (4-6). Thrombosis, which can occur in veins and arteries, is the most frequent complication of PNH and has a high mortality rate. Venous thrombosis can occur in the majority of organs, including the liver, lung, brain, kidney, spleen and bowel (7). A previous study demonstrated that a number of factors contributed to thrombosis in patients with PNH, including free hemoglobin, nitric oxide (NO) depletion, damaged endothelial cells, deregulation of the fibrinolytic system and platelet activation (8). However, the mechanism of thrombosis in patients with PNH is complex and remains unclear.

The glycoprotein CD177 antigen (NB1) is a GPI-linked protein that belongs to the lymphocyte antigen 6 superfamily, which also includes CD59 (9,10). NB1 was first reported in isoimmune neonatal neutropenia (11), but was later identified in transfusion-related acute lung injury, myeloproliferative neoplasms, gastric cancer and Wegener's granulomatosis associated with vasculitis (12-16). NB1 binds to platelet endothelial cell adhesion molecule to promote neutrophil migration and is involved in the inflammatory response (17,18). Proteinase 3 (PR3) is a neutrophil-derived serine proteinase that is primarily stored in azurophil granules in polymorphonuclear leukocytes. PR3 degrades a variety of matrix proteins, including fibronectin, laminin, vitronectin and collagen type IV (19), and regulates platelet activation through the cleavage and inactivation of proteinase-activated receptor 1 (PAR1), which is expressed on plasma membranes and is associated with NB1 (20,21). The present study aimed to investigate the expression of PR3 and NB1 in neutrophils, and explore the association between PR3 and thrombosis in patients with PNH or PNH-aplastic anemia (AA) syndrome.

Patients and methods

Patients. A total of 21 patients with classical PNH, 6 patients with PNH-AA syndrome and 25 healthy controls were enrolled in the present study. The patients with PNH and PNH-AA syndrome consisted of 14 males and 13 females, with median age of 29 years old (range, 21-43 years). All patients were recruited from the Department of Hematology of Tianjin Medical University General Hospital (Tianjin, China) between November 2014 and February 2016, and diagnosed according to the criteria for PNH set out by the Chinese Medical Association (22). Table I presents the clinicopathological characteristics of the patients included in the present study. There were 7 newly diagnosed patients (6 with PNH and 1 with PNH-AA). All patients exhibited the typical clinical manifestations of PNH and an abnormal expression of CD59 ($CD59^-$ granulocytes were >10% of total granulocytes), as detected by flow cytometry. Thrombosis was investigated by spiral computed tomography, magnetic resonance imaging or Doppler ultrasound wherever appropriate. A total of 2 patients had cerebral embolisms and 3 patients had portal vein thrombosis, in which 1 patient also had lower limb venous thrombosis.

All patients were treated with corticosteroids (prednisone 0.5 mg/kg/day, oral administration; Zhejiang Xianju Pharmaceutical Co., Ltd., Zhejiang, China) and vitamin E (300 mg/day, oral administration; Hebei Tiancheng Pharmaceutical Co., Ltd., Hebei, China) if they exhibited hemolysis. Patients with PNH-AA also received cyclosporine (3 mg/kg/day, oral administration; Huabei Pharmaceutical Co., Ltd., Huabei, China) for ≥6 months. A total of 5 patients with thrombosis were treated with low molecular weight heparin (0.1 ml/10 kg/day for 7-14 days, subcutaneous injection; Qilu Pharmaceutical Co., Ltd., Qilu, China) and warfarin (2.5-5 mg/day for 6-12 months, oral administration; Qilu Pharmaceutical Co., Ltd.).

The healthy controls consisted of 15 healthy donors and 10 patients with iron deficiency anemia, 13 males and 12 females, with a median age of 31 years (range, 27-58 years). The present study was approved by the Ethical Committee of Tianjin Medical University (Tianjin, China). Written informed consent was obtained from the patients for the publication of the present study.

Flow cytometry. Fresh peripheral blood (100 µl) was collected in EDTA-anticoagulation tubes and incubated with 20 µl of antibodies directed against CD59 [conjugated with phycoerythrin (PE); 1:5; cat no. 555764; BD Pharmingen; BD Biosciences, San Diego, CA, USA], NB1 (conjugated with allophycocyanin; 1:5; cat no. ab77230) and PR3 [conjugated with fluorescein isothiocyanate (FITC); 1:5; cat no. ab65255; both Abcam, Cambridge, MA, USA]. Their isotype control antibodies (1:5; BD Biosciences) were used as the negative controls. Following incubation in the dark for 30 min at room temperature, red blood cells were lyzed with 10 ml erythrocytolysin solution (BD Biosciences) and then centrifuged at room temperature at 150 x g for 5 min. The cells were then washed twice with PBS. Finally, the cells were resuspended in 300 ul PBS. Flow cytometry was performed using a BD FACSCalibur™ Flow Cytometer (BD Biosciences) and ≥20,000 events were acquired for each sample. All results were analyzed using CellQuest™ Pro Software 4.0.2 (BD Biosciences).

ELISA. The levels of PR3, NB1 and PAR1 in the serum were measured by ELISA. Briefly, 100 µl of diluted (1:100) capture antibodies directed against PR3 (Human proteinase-antineutrophil cytoplasmic antibody; PR3-ANCA ELISA kit; cat no. fk1344Y; R&D Systems, Inc, Minneapolis, MN, USA), NB1 (CD177 ELISA kit; cat no. EH1752; Cusabio Biotech Co., Ltd., Wuhan, China) or PAR1 (Human Protease Activated Receptor 1 ELISA kit; cat no. SEC939Hu; Cloud-Clone Corp., Katy, TX, USA) were added to each well and the plates were incubated at 4˚C overnight. The plates were washed three times, then 200 µl assay diluent was added to each well, and the plates were incubated for 1 h at room temperature. The plates were then washed three times, and diluted standards and the sera (100 µl) of the patients and controls were added to the wells in duplicate, after which the plates were incubated for 2 h at room temperature. Following another wash, 100 µl of a diluted (20 ng/ml) working detector was added to each well and the plates were incubated for 1 h at room temperature. The wells were washed seven times. Then, 3,3',5,5'-tetramethylbenzidine 1:100 substrate solution was added to each well and the samples were incubated in the dark at room temperature for 30 min. Stop solution was added and the optical density at 450 nm was measured within 30 min using a VersaMax™ ELISA Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Neutrophil isolation. Neutrophils were isolated from the peripheral blood of patients with PNH/PNH-AA and healthy controls. Briefly, 5 ml peripheral blood was collected into a tube containing 2 mM EDTA. Then, the blood was layered over a Ficoll Paque Plus solution (cat no. 17-1440-02; GE Healthcare, Chicago, IL, USA) and centrifuged at room temperature for 20 min at 700 x g according to the manufacturer's protocol. Neutrophils were isolated from the buffy coat layer and washed with PBS without calcium or magnesium. The cells were then washed twice with PBS and centrifuged at room temperature at 200 x g for 10 min. If the neutrophil solution mixed with red blood cells, Red Blood Cell Lysis Buffer (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used to lyse the red blood cells before the neutrophils were quantified.

PNH clone sorting by magnetic-activated cell sorting (MACS). In 90 µl autoMACS Running buffer (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) 10,000,000 cells were resuspended, according to manufacturer's protocol. Then, 20 µl CD59-PE and 20 µl PE MicroBeads (Miltenyi Biotec GmbH) were added and the cells were incubated at 4˚C in the dark for 20 min. Following a wash with 2 ml of the buffer, the cells were centrifuged at room temperature at 300 x g for 5 min. The cells were then resuspended in 500 µl of the buffer. The LD column was placed in the magnetic field of a suitable Quadro MACS separator (both Miltenyi Biotec GmbH). Following the preparation of the column by rinsing it with 2 ml of the buffer, the cells were applied to the column. The flow through containing unlabeled cells was collected. Finally, the column was washed with 2 ml of the buffer. The purity of the PNH clone ($CD59^-$ cells) was detected by flow cytometry.

Table I. Clinicopathological characteristics of the patients included in the present study.

Patient no.	Age (years)	Gender	Diagnosis	PNH clone granulocytes (%)	WBC (x10^9/l)	Hb (g/l)	PLT (x10^9/l)	RET (%)
1	38	F	PNH-AA	19.33	5.96	106	113	5.15
2	43	F	PNH-AA	30.23	3.56	98	110	6.12
3	21	F	PNH-AA	20.83	3.30	87	80	5.40
4[b]	55	M	PNH-AA	33.43	2.70	70	254	6.01
5	26	M	PNH	50.68	4.58	133	80	0.80
6	13	F	PNH	88.84	3.34	91	42	7.84
7	33	F	PNH	63.21	7.27	81	223	9.16
8[a,b]	25	F	PNH	85.46	4.73	76	71	7.88
9	25	F	PNH	78.11	2.88	83	92	6.70
10[a,b]	24	F	PNH	97.98	1.34	67	36	4.95
11[a]	58	M	PNH	86.75	3.62	114	38	3.92
12	50	F	PNH	45.54	4.20	85	123	5.70
13	46	F	PNH	62.32	5.50	87	219	4.20
14	24	F	PNH	57.88	3.90	84	111	3.80
15[a,b]	20	M	PNH	92.57	3.91	76	51	3.37
16	46	M	PNH-AA	31.23	3.55	111	75	5.35
17[a,b]	27	F	PNH	59.41	1.40	50	28	4.90
18	11	M	PNH	55.60	6.10	78	102	5.10
19	27	M	PNH	66.38	19.34	110	68	4.26
20[b]	29	M	PNH	93.60	5.90	61	78	8.50
21	15	M	PNH-AA	10.98	4.80	108	310	1.10
22[b]	43	M	PNH	56.37	10.59	63	56	8.36
23	33	M	PNH	80.64	10.32	89	92	7.76
24	16	F	PNH	92.00	7.69	98	158	10.30
25	22	M	PNH	50.34	7.10	79	88	5.89
26	45	M	PNH	87.11	5.60	87	77	5.21
27	32	M	PNH	98.20	6.10	91	67	8.90

[a]Combined with thrombosis (8 and 11, cerebral embolism; 10 and 15, portal vein thrombosis; 17, portal vein thrombosis combined lower limbs venous thrombosis). [b]Newly diagnosed patients. PNH, paroxysmal nocturnal hemoglobinuria; AA, aplastic anemia; M, male; F, female; WBC, white blood cells; Hb, hemoglobin; PLT, platelets; RET, reticulocytes.

Immunofluorescence (IF). The sorted PNH cells were collected for smear. The cells were smeared on a coverslip and then the smear was rinsed with PBS three times (5 min each) and blocked with 1% BSA (Sigma-Aldrich; Merck KGaA) at room temperature for 30 min. Then the coverslips were incubated with anti-PR3 (1:50; cat no. ab65255) and anti-NB1 (1:50; cat no. ab26013; both Abcam) antibodies conjugated with FITC at 4˚C overnight and washed with PBS three times. Then, the coverslips were stained at room temperature with hematoxylin for 1 min and rinsed with ammonium hydroxide. Following the coverslips being washed two times with PBS (3 min each), the coverslips were blocked for 3 days at room temperature, with glycerin and viewed under an oil immersion lens (magnification, x1000; Olympus Corporation, Tokyo, Japan).

Isolation of total RNA and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from the neutrophils using TRIzol™ reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). From the purified RNA, 1 μg was used for the RT-qPCR analysis using the SuperScript™ First-Strand Synthesis system for RT-PCR (Invitrogen; Thermo Fisher Scientific, Inc.). The RT-qPCR was performed using SYBR® *Premix Ex Taq*™ (Tli RNaseH Plus), ROX plus and the Thermal Cycler Dice Real Time system (both Takara Bio, Inc., Otsu, Japan) in a 96-well plate according to the manufacturer's protocol. The amplification utilized 45 cycles at 95˚C for 30 sec and 56.7˚C for 30 sec, with the extension at 72˚C for 30 sec. The primers used for the RT-qPCR were as follows: PR3 forward (F), 5'-ACGCGG AGAACAAACTGAAC-3' and reverse (R), 5'-AGGGACGAA AGTGCAAATGT-3'; and NB1 F, 5'-GCAGAGACTTCAGGG TGCTC-3' and R, 5'-CGACACATTTCTAACGACACG-3'. Human β-actin was used as a housekeeping gene for quantity normalization with the following primer sequences: F, 5'-CTG GAACGGTGAAGGTGACA-3' and R, 5'-AAGGGACTTCCT GTAACAATGCA-3'. The PR3 and NB1 levels were calculated using the $2^{-\Delta\Delta Cq}$ method (23) following normalization of the data.

Western blotting. Isolated neutrophils were lysed in RIPA buffer (R&D Systems, Inc., Minneapolis, MN, USA) supplemented with complete protease inhibitor and phosphatase inhibitors (both Roche Diagnostics, Basel, Switzerland). Protein levels were detected using bicinchoninic acid assay kit (Thermo Fisher Scientific, Inc.). A total 40 μg protein/lane were separated by SDS-PAGE on a 12% gel and transferred to nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% milk (BD Biosciences) for 1 h at room temperature, followed by incubation with primary antibodies, anti-PR3 (1:50; cat no. ab65255, Abcam) and GAPDH (1:1,000; Anti-GAPDH Monoclonal Antibody; cat no. A01020; Abbkine Scientific Co., Ltd., Wuhan, China) at 4˚C overnight. The membranes were washed with Tris-buffered saline with Tween-20 (20 mM Tris-HCl buffer, pH=7.4, containing 150 mM NaCl and 0.05% Tween-20) three times and then incubated with secondary antibodies horseradish peroxidase-labeled goat anti-mouse Immunoglobulin G (1:2,500; cat no. ab6789; Abcam) at room temperature for 2 h. The reaction was detected with Super ECL Plus Detection Reagent (Thermo Fisher Scientific, Inc.). Protein levels were normalized to GAPDH.

Statistical analysis. Results for each group are expressed as the median (serum level of D-Dimer) or mean ± standard error of the mean (PR3, NB1 and PAR1 levels). Statistical analysis was performed using one-way analysis of variance followed by a Dunnett's post hoc test. The t-test was performed for two groups. Correlations between different percentages of PR3 and all variables was determined using Spearman's correlation coefficient. Data were analyzed using GraphPad Prism software (version 5.0; GraphPad Software, San Diego, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

PR3 and NB1 expression on the neutrophil plasma membranes of patients with PNH/PNH-AA is decreased but not correlated. The mRNA levels of PR3 and NB1 were detected in 27 patients with PNH/PNH-AA and 25 healthy controls by flow cytometry (Fig. 1A and B). The expression of NB1 on $CD59^-$ neutrophils ($CD59^-NB1^+/CD59^-$) in patients with PNH/PNH-AA (20.61±26.07%) was significantly lower compared with the $CD59^+$ neutrophils ($CD59^+NB1^+/CD59^+$) in patients with PNH/PNH-AA (72.25±25.62%, P=0.001) and healthy controls (67.72±19.6%, P=0.001) (Fig. 1C1). The expression of PR3 on $CD59^-$ neutrophils ($CD59^-PR3^+/CD59^-$) in patients with PNH/PNH-AA (70.40±29.86%) was significantly lower compared with the healthy control group (93.28±10.53%, P=0.001) and $CD59^+$ neutrophils ($CD59^+PR3^+/CD59^+$) in patients with PNH/PNH-AA (85.68±22.21%, P=0.011) (Fig. 1C2). The expression of PR3 in the two latter demonstrated no significant differences (P=0.252).

Notably, PR3 mRNA expression did not correlate with NB1 mRNA expression (r=0.194; P=0.393; Fig. 1C3), indicating that PR3 expression is not associated with NB1. In order to explore the association between PR3 and NB1, their expression levels on $CD59^-$ neutrophils were detected by IF. The results demonstrated that PR3 was partially expressed in patients with PNH/PNH-AA (Fig. 1D), whereas NB1 was not expressed in patients with PNH/PNH-AA due to a defect in the GPI anchor (Fig. 1E).

PR3 in the serum of patients with PNH/PNH-AA is decreased, which is negatively correlated with PAR1 and D-Dimer levels. PAR1 and D-Dimer are associated with thrombosis, thus their protein levels were investigated. The serum level of PAR1 in patients with PNH/PNH-AA (1.38±0.96 μg/l) was significantly higher compared with that in the healthy controls (0.47±0.29 μg/l) (P<0.001; Fig. 2A). The serum level of PR3 in patients with PNH/PNH-AA (2,262.72±802.80 pg/ml) was significantly lower compared with that in the healthy controls (3,292.92±651.68 pg/ml) (P<0.0001; Fig. 2B). However, the serum level of NB1 in patients with PNH/PNH-AA (3.881±0.1663 ng/ml) demonstrated no significant difference compared with that in the healthy controls (3.840±0.1188 ng/ml) (P=0.2007l; Fig. 2C).

The median level of D-Dimer in patients with PNH/PNH-AA (511 ng/dl) was significantly higher compared with the healthy controls (343 ng/dl) (P=0.04; data not shown). Furthermore, the level of D-Dimer between the patients with thrombosis (Table II) and without thrombosis was compared. The results revealed that the median level of D-Dimer in the 5 patients with thrombosis (2,104 ng/dl) was significantly increased compared with those without thrombosis (226 ng/dl) (P=0.001; data not shown). In addition, the levels of D-Dimer (r=-0.503; P=0.028; Fig. 2D) and PAR1 (r=-0.456; P=0.043; Fig. 2E) were significantly negatively correlated with the level of PR3.

PNH clones exhibit no significant difference in mRNA and protein levels of PR3 and NB1 compared with neutrophils from the healthy controls. $CD59^-$ neutrophils were sorted by MACS following their isolation from the neutrophils of the patients. The purity of $CD59^-$ cells, sorted by MACS (Fig. 3A, B), was >85%. The expression of PR3 and NB1 in $CD59^-$ cells in the patients and controls ($CD59^+$ cells) was then analyzed by RT-qPCR and western blot analyses.

The mRNA level of PR3 in patients with PNH/PNH-AA (1.344±0.3679) demonstrated that there was no significant difference compared with the controls (1.815±0.5005) (P=0.4439; Fig. 3C). Similarly, the mRNA level of NB1 in the patients with PNH/PNH-AA (1.826±0.4010) and the healthy controls (1.485±0.3563) demonstrated no significant difference (P=0.5359; Fig. 3D). The western blotting results revealed that there was no marked difference in PR3 protein levels between patients with PNH/PNH-AA and the healthy control group (Fig. 3E).

Discussion

Thromboembolism is the primary cause of mortality in patients with PNH and usually occurs in the hepatic veins, which leads to Budd-Chiari syndrome, the cerebral veins and sinuses. Thus far, the mechanism of thrombosis in PNH has been unclear. NO synthesis in endothelial cells maintains normal flow of blood and inhibits platelet aggregation. In patients with PNH, extensive intravascular hemolysis results in the production of large amounts of free hemoglobin in plasma. The free hemoglobin

Table II. PR3, NB1 and D-Dimer serum levels in patients with paroxysmal nocturnal hemoglobinuria combined with thrombosis.

Patient no.	G, $CD59^-$ (%)	D-Dimer level (ng/dl)	$CD59^-PR3^+$/ $CD59^-$ (%)	$CD59^+PR3^+$/ $CD59^+$ (%)	$CD59^-NB1^+$/ $CD59^-$ (%)	$CD59^+NB1^+$/ $CD59^+$ (%)
8	85.46	830	24	25	11	81
10	97.98	2,104	56	91	16	80
11	86.75	1,355	79	86	1	13
15	92.57	5,324	99	99	0.9	93
17	59.41	5,835	96	98	0.5	69

G, granulocytes; CD, cluster of differentiation; PR3, proteinase 3; NB1, CD177 antigen.

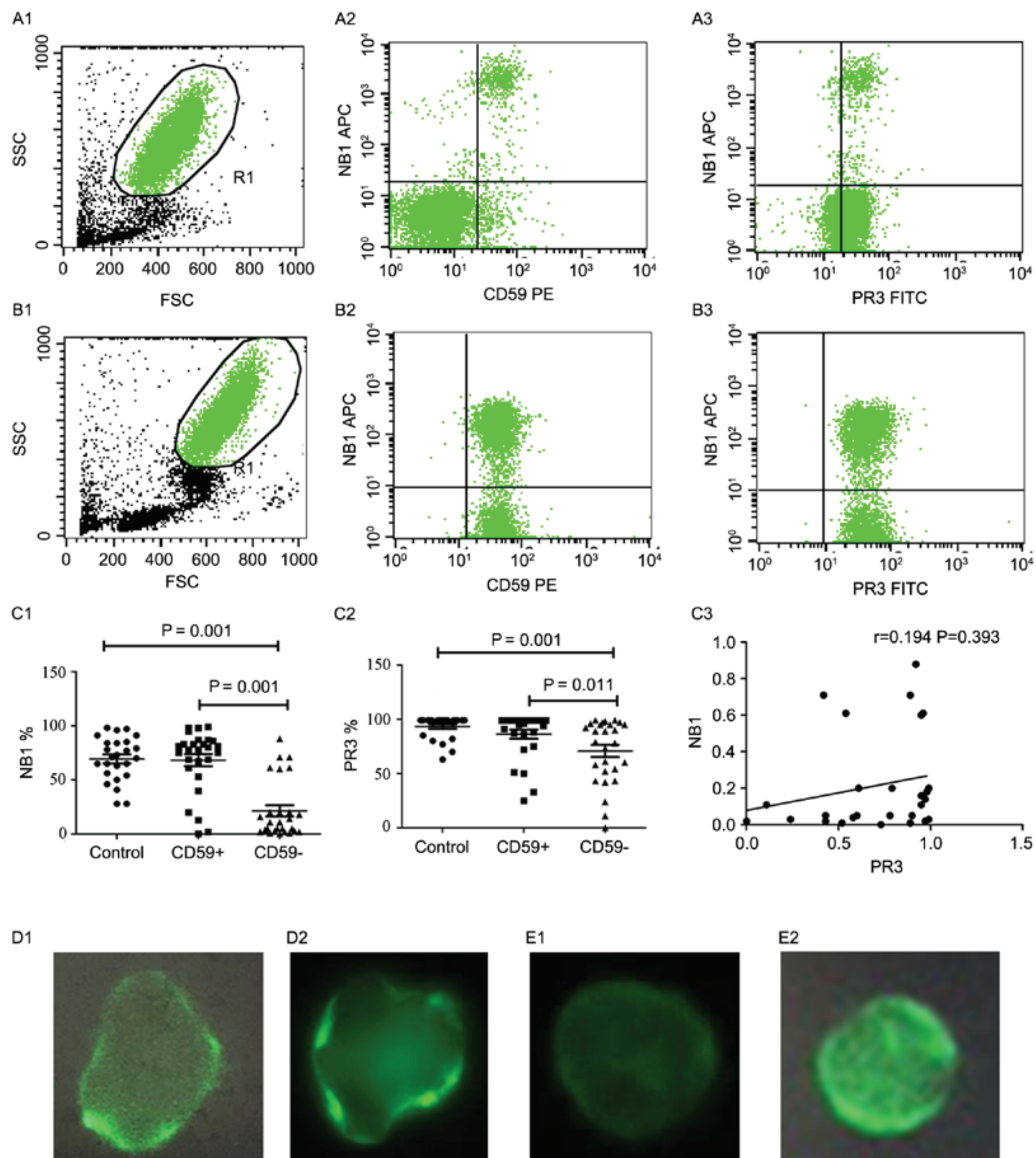

Figure 1. PR3 and NB1 expression on the neutrophil plasma membranes of patients with PNH or PNH-AA is decreased. The expression of PR3 and NB1 were detected by flow cytometry in (A) PNH/PNH-AA patients and (B) healthy controls (presented as the mean ± standard error of the mean). (A1 and B1) The neutrophils were gated as R1 and then (A2 and B2) NB1 expression was investigated on $CD59^-$/$CD59^+$ neutrophils. (A3 and B3) PR3 and NB1 were demonstrated to be expressed on $CD59^-$/$CD59^+$ neutrophils. (C) Quantification and analysis of the flow cytometry results. (C1 and C2) The results demonstrated that the expression of NB1 and PR3 on $CD59^-$ neutrophils significantly decreased compared with $CD59^+$ neutrophils in patients with PNH/PNH-AA and the healthy controls. (C3) No correlation was identified between PR3 and NB1 expression in patients with PNH/PNH-AA. Furthermore, the expression of these two proteins were measured by immunofluorescence. PR3 was partially expressed on $CD59^-$ neutrophils of (D1) patients with PNH/PNH-AA compared with (D2) healthy controls, while no NB1 expression was identified on $CD59^-$ neutrophils of (E1) patients with PNH/PNH-AA compared with (E2) healthy controls. SSC, side-scattered light; FSC, forward-scattered light; CD, cluster of differentiation; NB1, CD177 antigen; APC, allophycocyanin; PE, phycoerythrin; PR3, proteinase 3; FITC, fluorescein isothiocyanate; PNH, paroxysmal nocturnal hemoglobinuria; AA, aplastic anemia.

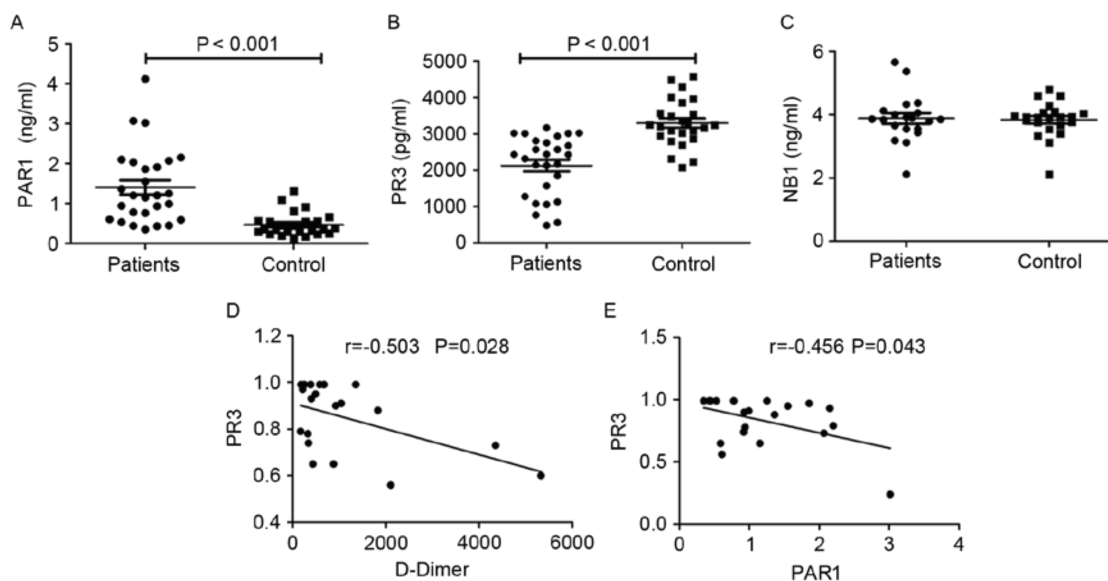

Figure 2. PR3 in the serum of patients with PNH/PNH-AA is decreased, and negatively correlated with PAR1 and D-Dimer levels. In patients with (A) PNH the level of PAR1 increased, while (B) PR3 decreased (presented as the mean ± standard error of the mean). (C) No significant change in NB1 expression was identified in patients with PNH/PNH-AA compared with healthy controls. The level of PR3 negatively correlated with (D) D-Dimer (presented as the median) and (E) PAR1 levels in patients with PNH/PNH-AA. PAR1, proteinase-activated receptor 1; PR3, proteinase 3; NB1, cluster of differentiation 177 antigen; PNH, paroxysmal nocturnal hemoglobinuria; AA, aplastic anemia.

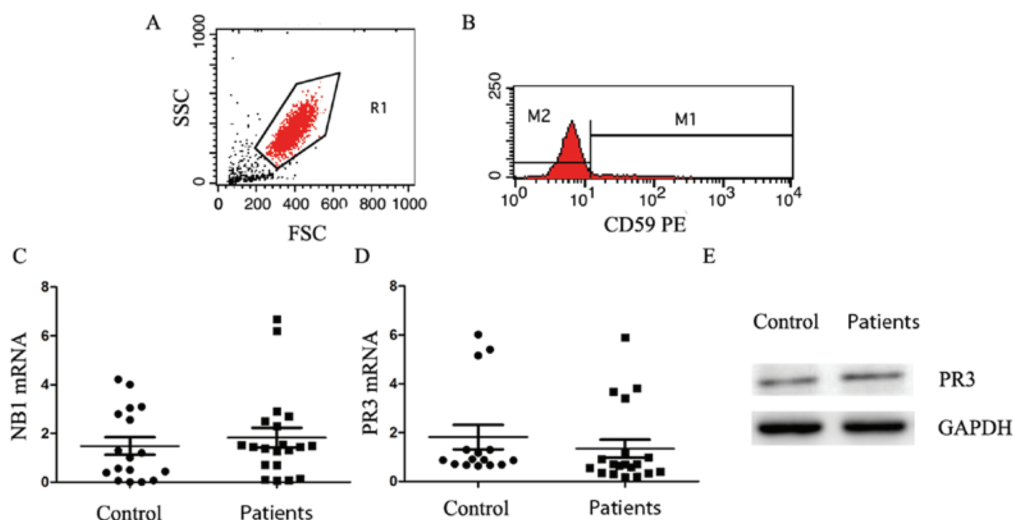

Figure 3. PNH clones exhibit no significant difference in mRNA and protein levels of PR3 and NB1 compared with neutrophils from the controls. (A) The neutrophils were isolated from the blood of patients with PNH/PNH-AA; the purity, detected by flow cytometry, was >85%. (B) $CD59^-$ neutrophils were sorted by magnetic-activated cell sorting and the purity was determined to be >90%. No significant differences in the mRNA expression of (C) PR3 or (D) NB1 were identified between patients with PNH/PNH-AA and the healthy controls (presented as the mean ± standard error of the mean). (E) No notable differences in PR3 protein expression were identified between patients with PNH/PNH-AA and the healthy controls. SSC, side-scattered light; FSC, forward-scattered light; CD, cluster of differentiation; NB1, CD177 antigen; PE, phycoerythrin; PR3, proteinase 3; PNH, paroxysmal nocturnal hemoglobinuria; AA, aplastic anemia.

serves a role as a NO effective scavenger in combination with NO, and NO is depleted, resulting in platelet aggregation and activation (24,25). Another factor associated with thrombosis is urokinase-type plasminogen activator receptor (uPAR), a GPI-linked protein expressed on neutrophils that mediates endogenous thrombolysis through a urokinase-dependent mechanism (26-28). Sloand *et al* (29) demonstrated that in patients with PNH, membrane GPI-anchored uPAR is decreased or absent on granulocytes and platelets, while soluble uPAR (suPAR) levels are increased in patients' plasma. Increased levels of su-PAR compete with urokinase receptors on the cell membrane, reducing plasmin production, thereby reducing fibrinolytic activity and promoting thrombosis and stabilization. A previous study demonstrated that the adhesion and aggregation of platelets was compensatively decreased in patients with PNH, particularly in $CD59^+$ platelets (30).

The present study aimed to explore the expression of PR3 and its effect on thrombosis in patients with PNH. Several studies have suggested that there is an association between NB1 and PR3, which are co-expressed on the plasma membrane of the same subset of neutrophils; these studies indicated that NB1 is a receptor of PR3 (16,31-33). However, Hu *et al* (34,35)

demonstrated that neutrophils from NB1 negative individuals expressed low levels of PR3 following priming with tumor necrosis factor αumor necrosis factor low leis not an exclusive binding partner of PR3. The flow cytometry results in the present study demonstrated that PR3 and NB1 expression decreased on $CD59^-$ neutrophils due to a lack of GPI-linked proteins; however, there was no correlation between PR3 and NB1 expression. In addition, the IF results demonstrated that PR3 was partially expressed on the $CD59^-$ neutrophils of patients with PNH, while there was no NB1 expression. A hypothesis for the low expression of PR3 on $CD59^-$ neutrophils from patients with PNH compared with $CD59^+$ neutrophils from patients with PNH and normal controls may be that PR3 binds to other receptor(s) to exert its function.

Furthermore, the level of PR3 in serum was identified to be significantly decreased in patients with PNH/PNH-AA, and negatively correlated with PAR1 and D-Dimer levels. PR3 can degrade PAR1, causing inhibition of active thrombin and regulating platelet activation (21). PAR1 combines with thrombin to induce thrombosis. Thrombin binding to PAR1 on platelets induces platelet activation and strengthens platelet adhesion in order to promote the aggregation of platelets and thus cause thrombosis (36,37). Another study demonstrated that PR3 induced platelets to change shape via the Rho/Rho kinase and Ca^{2+} signaling pathways (38). The results of the present study indicated that lower PR3 level in serum of patients with PNH/PNH-AA resulted in increased PAR1 level, and thus an increased concentration of activated platelets. To investigate the hypothesis of NB1 regulation of PR3 expression, RT-qPCR analysis was performed. No significant difference in PR3 and NB1 mRNA levels was identified between the patients with PNH/PNH-AA and the control group. Thus, this hypothesis was not validated.

In conclusion, the expression of PR3 on the plasma membrane of neutrophils decreased in patients with PNH/PNH-AA, but was still partially expressed. The level of PR3 in the serum of patients with PNH/PNH-AA also decreased, which lead to an increase in PAR1 expression, indicating increased platelet activation. However, the mechanism regulating PR3 expression in patients with PNH requires further exploration in the future.

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