Prospective study of thrombosis and thrombospondin-1 expression in Chuvash polycythemia

In Chuvash polycythemia (CP), homozygous germline *VHL*^{R200W} results in augmented hypoxia sensing, elevated erythropoietin and hemoglobin, and increased morbidity and mortality from thrombosis, but the relative risks and molecular basis have not been prospectively evaluated.[1-3] We enrolled 128 CP adults and 128 controls from Russia's Chuvash Republic in an observational study from 2005-2009, in order to prospectively define the risk of complications and to provide mechanistic insights. We hypothesized that variation in the expression of hypoxia inducible factor (HIF)-regulated genes may contribute to increased thrombosis. During a median follow up of 8.8-years, CP was associated with a 9.6-fold increase in the rate of new thrombosis compared to controls, after adjustment for significant risk factors such as age and smoking. The probability of new thrombosis in CP did not increase with higher baseline hemoglobin concentration, but it increased with age, smoking, baseline therapeutic phlebotomy and higher expressions of *THBS1, CXCL2* and *EREG*.

Hypomorphic VHL^{R200W} impairs HIF-α degradation, leading to an increased transcription of many HIF-regulated genes, including erythropoietin,[1-3] and to elevated hematocrit levels, thrombosis and early mortality, but not malignancy.[2-4] Whether the increased risk of thrombosis is related to the elevated hemoglobin concentration has been unclear,[2] and hypoxia itself has been implicated as a risk for thrombosis.[5] The British Committee for Standards in Haematology recommends phlebotomy for polycythemia vera if the hematocrit is >45%, and for idiopathic erythrocytosis/polycythemia if the hematocrit is >54%,[6] but its benefits for CP are unknown.

The CP patients and controls in this study were matched by age, sex and place of residence within Russia's Chuvash Republic, and underwent *VHL*^{R200W} genotyping.[2] The ethics committees of the Chuvash State University and the Republic Clinical Hospital gave their approval. Written informed consent was obtained from all subjects according to the Declaration of Helsinki. Consistent with previous studies, CP adults had higher hemoglobin and erythropoietin concentrations, lower systemic blood pressure, body mass index, white blood cell count, and serum ferritin concentration, less history of hypertension and more reports of bleeding (Table 1).[2] They were also more likely to be current smokers than controls. Twenty-seven patients aged 24-76 years and 3 controls aged 45-67 years had a history of thrombosis. CP patients with past thrombosis were on average 10 years older than those without (*P*=0.002).

The subjects were followed up between 2015 and 2016

Table 1. Baseline characteristics of adult Chuvash polycythemia (CP) patients and controls at time of enrollment in the case-control study. Results in median (interquartile range) or n (%); comparison with the Kruskal-Wallis test for continuous and Pearson's χ^2 test for categorical variables.

	CP		Controls		
	N	Result	N	Result	*P*
Age (years)	128	38 (26-50)	128	40 (26-50)	0.4
Female sex	128	70 (54.7%)	128	68 (53.1%)	0.8
History of phlebotomy therapy	128	100 (78.1%)	128	0 (0%)	<0.001
History of smoking	128	41 (32.0%)	128	23 (18.0%)	0.009
History of thrombosis*	128	27 (21.1%)	128	3 (2.3%)	<0.001
History of bleeding	128	14 (10.9%)	128	2 (1.6%)	0.002
History of hypertension	128	8 (6.3%)	128	27 (21.1%)	0.001
History of diabetes mellitus	128	3 (2.3%)	128	1 (0.8%)	0.3
History of malignancy**	128	2 (1.6%)	128	1 (0.8%)	0.6
BMI (kg/m^2)	128	21.5 (19.8-23.7)	128	23.3 (21.1-26.3)	<0.001
Mean arterial pressure (mm Hg)	128	91 (85-97)	128	94 (88-102)	0.009
Erythrocytes (x10^3/uL)	127	6.45 (6.00-7.19)	127	4.69 (4.22-4.99)	<0.001
Hemoglobin (g/dL)	127	17.9 (15.9-19.8)	128	13.8 (12.6-15.0)	<0.001
Hematocrit (%)	115	53.4 (47.7-58.5)	127	40.3 (37.2-43.4)	<0.001
MCV (fL)	110	80.7 (74.0-87.0)	127	87.7 (84.3-90.8)	<0.001
MCH (g/dL)	119	27.3 (23.7-30.9)	128	29.9 (28.4-31.2)	<0.001
MCHC (g/dL)	111	33.2 (31.3-35.0)	127	34.2 (33.0-35.3)	0.001
White blood cells (per uL)	127	5.70 (4.60-7.12)	128	6.40 (5.34-7.46)	0.001
Neutrophils (per uL)	92	3.03 (2.07-3.79)	122	3.52 (2.95-4.47)	<0.001
Lymphocytes (per uL)	98	1.90 (1.55-2.32)	126	2.19 (1.83-2.54)	0.003
Platelets (per uL)	127	219 (165-268)	128	247 (209-300)	<0.001
Erythropoietin (U/L)	89	48.6 (24.4-88.3)	44	8.9 (7.3-13.8)	<0.001
Serum ferritin (ug/L)	86	11 (6-23)	43	53 (23-105)	<0.001

*Twenty-seven CP patients had a history of 40 thromboses- 18 with 1 thrombosis, 5 with two thromboses, 4 with three thromboses: stroke (n=10), myocardial infarction (n=10), splanchnic thrombosis (n=9), deep vein thrombosis (n=8), pulmonary embolism (n=3). Three controls had a history of a single thrombosis: stroke, pulmonary embolism, myocardial infarction. **History of breast carcinoma and Hodgkin lymphoma in CP patients; history of breast carcinoma in a control. BMI: body mass index; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration.

at a range of 6.2-10.7 years after enrollment. Thirty CP patients (23.4%) experienced a new thrombosis at a median age of 50 (21-75) years and 3 controls (2.3%) developed a new thrombosis at a median age of 80 (45-82) years. Four patients also experienced an additional thrombosis for a total of 34 new thromboses: myocardial infarction (n=10), splanchnic thrombosis (n=8), stroke (n=7), lower extremity or pelvic venous thrombosis (n=3), pulmonary embolism (n=3), lower extremity arterial thrombosis (n=2), and sudden death from suspected stroke (n=1). The controls experienced stroke (n=2) and myocardial infarction (n=1). In multivariate Cox proportional hazards analysis that included variables with univariate significance, the rate of new thrombosis was not elevated with increasing hemoglobin concentration (hazards ratio [HR] 0.9, 95% confidence interval [CI] 0.8-1.1, *P*=0.4), but it was higher with CP diagnosis (HR 9.6, 95% CI 2.6-35.4, *P*=0.001; Figure 1A), past thrombosis (ratio 1.9 for each event, 95% CI 1.3-2.7, *P*=0.001), age (ratio 1.5 for a 10-year increase, 95% CI 1.1-2.0, *P*=0.005), and current smoking status (ratio 2.5, 95% CI 1.1-5.6, *P*=0.023). The prospective rate of new thrombosis in our relatively young cohort of CP patients (median age 38 years at enrollment, 0.031 events/patient per year) is more than twice that of a German registry of 438 myeloproliferative neoplasm patients with 8 years of follow up (median age 60 years at diagnosis, 0.014 events/patient per year).[7]

Nine (7.0%) CP patients died during follow up compared to 2 (1.6%) controls (HR 4.5, 95% CI 0.95-20.5, *P*=0.058). The median age at death was 54 years (range 21-75) in the patients and 81 years (80-81) in the controls; all deaths were related to thrombosis. Four patients and no controls reported bleeding complications during follow up, usually menstrual or gastrointestinal. One control developed a new malignancy (colorectal carcinoma).

We also assessed new thrombosis in analyses restricted to CP subjects, who were categorized based on phlebotomy history at study entry as follows: no phlebotomy (n=27), remote phlebotomy (>1 year before enrollment; n=36), and recent phlebotomy (<1 year before enrollment; n=65). The serum ferritin concentration was lower in those with a history of phlebotomy after adjustment for age, sex and bleeding history (geometric means of 10 *versus* 22 ug/L, *P*=0.023). By multivariate Cox proportional hazards analysis, increasing age, past history of thrombosis, current cigarette smoking, phlebotomy categories and treatment with pentoxifylline, a non-selective phosphodiesterase inhibitor used to improve blood flow, had significant associations with new thrombosis (Table 2). Phlebotomy is considered to be a cornerstone of therapy for polycythemia vera, but when the Polycythemia Vera Study Group compared phlebotomy, radioactive phosphorus and chlorambucil, there was an increased risk of stroke within 24 hours of phlebotomy.[8] Phlebotomy leads to iron deficiency, which

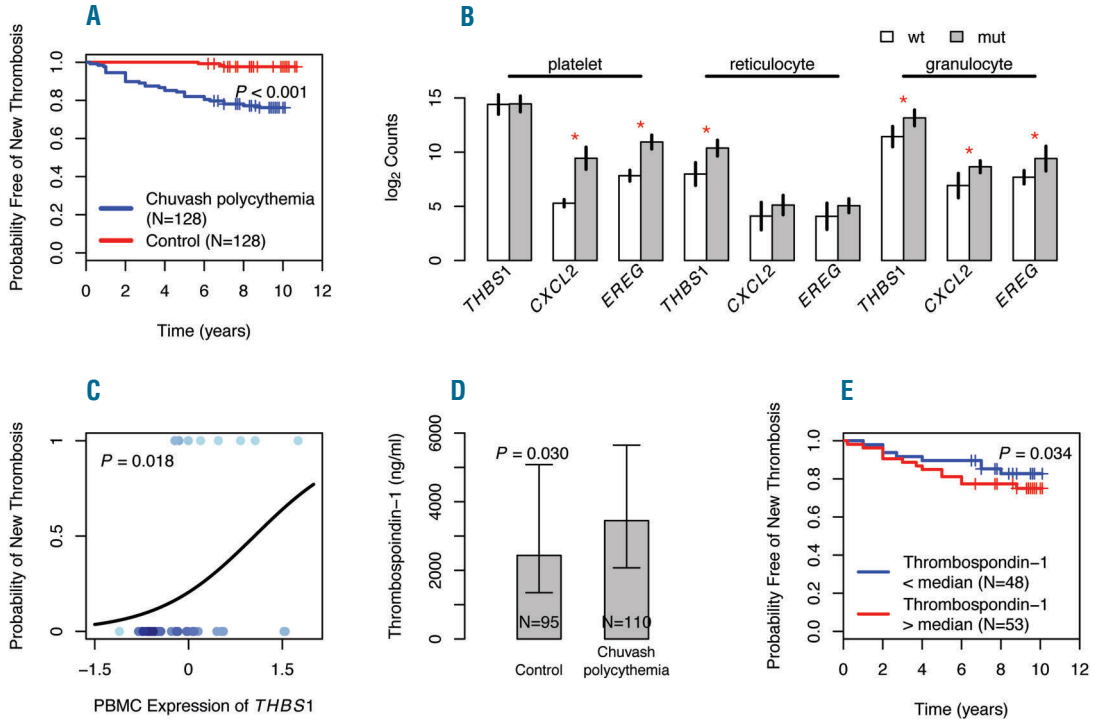

Figure 1. Thrombosis and *THBS1* expression in Chuvash polycythemia (CP) patients. A) Comparison of incidence of thrombosis in CP subjects *versus* controls. Prospective evaluation of new thrombosis in 128 CP subjects and 128 controls matched by age, sex and residence over a median of 8.8 years. The rate of new thrombosis was higher in CP subjects (hazards ratio 12.7). B) Mean and standard deviation of normalized $\log_2$ counts from RNA-seq data for *THBS1*, *CXCL2* and *EREG* in control (wt) and CP (mut) individuals among platelets, reticulocytes, and granulocytes. Comparison between controls and CP patients with nominal *P*<0.05 is indicated by an asterisk. C) The probability of new thrombosis against the relative PBMC expression of *THBS1* in CP patients, with a curve of prediction based on the logistic regression model. Depth of color shows the density of data points. D) Plasma thrombospondin-1 concentrations are greater in CP individuals than controls: median (interquartile range) of 3,448 (2,073-5,648) *versus* 2,434 (1,350-5,081) ng/ml. E) Among CP subjects with a history of less than two past thromboses, those with plasma thrombospondin-1 concentrations above the median of 3,448 ng/ml have a higher rate of thrombosis than those with concentrations below the median. PBMC: peripheral blood mononuclear cells; wt: *VHL* wild-type; mut: VHL^{R200W} homozygote.

Table 2. Multivariate Cox Proportional Hazards Model of predictors of new thrombosis during follow up in 128 CP patients.

Variable	Hazards Ratio	95% Confidence Interval	P
Number of past thromboses (range from 0 to 3)	1.9	1.3-2.8	0.001
Treatment with pentoxifylline	3.3	1.5-7.2	0.003
Average number of cigarettes smoked per day in the past year (increments of 10)	1.9	1.1-3.3	0.018
Remote and recent phlebotomy categories	2.0	1.01-3.8	0.045
Age (increments of 10 years)	1.3	0.99-1.9	0.060

inhibits the PHD2 enzyme, increases HIF-α stability and further elevates HIF-regulated gene transcription,[3] and iron deficiency has an increased thrombotic risk.[9]

As the increased risk of thrombosis in CP could not be attributed directly to the raised hematocrit, we considered whether altered gene expression associated with the germline *VHL* mutation may be responsible for additional thrombotic risk. RNA was prepared from peripheral blood mononuclear cells (PBMCs),[3] purified granulocyte and platelet fractions,[10] and reticulocytes,[11] as previously described. Granulocyte, platelet and reticulocyte RNA underwent stranded library construction (Illumina TruSeq Stranded Total RNA Sample Preparation Kit with Ribo-Zero Human/Mouse/Rat), sequencing (HiSeq 125 Cycle Paired-End Sequencing v4) and analysis (STAR,[12] DESeq2[13]). We identified CP-induced genes in PBMCs (8 VHL^{R200W} homozygotes *versus* 17 wild-type individuals) by gene expression array, and in reticulocytes (5 VHL^{R200W} homozygotes *versus* 5 wild-type individuals), platelets (3 VHL^{R200W} homozygotes *versus* 4 wild-type individuals) and granulocytes (6 VHL^{R200W} homozygotes *versus* 8 wild-type individuals) by RNA sequencing (seq).

Six genes were upregulated in CP by ≥1.5-fold in PBMCs and at least 2 additional specific blood cell types: *THBS1, CXCL2, EREG, CD300E, IFIT3* and *MAFB* (Figure 1B). In 34 patients, we analyzed the correlation of baseline PBMC expression of the 6 genes[3] with new thrombosis (n=8) by logistic regression with adjustment for age.[14] Baseline PBMC expression of *THBS1* (OR=4.5, 95% CI: 1.3-24, *P*=0.018, multiple comparison adjusted *P*=0.11) (Figure 1C), *CXCL2* (OR=6.1, 95% CI: 0.95-58, *P*=0.057, adjusted *P*=0.17) and *EREG* (OR=3.0, 95% CI: 0.47-21, *P*=0.2, adjusted *P*=0.5) was associated with new thrombosis, and expression of all 3 genes showed a trend to be higher with baseline phlebotomy history (β=0.16-0.18, *P*=0.2) using linear regression adjusted for age. Thrombospondin-1, encoded by *THBS1*, is a glycoprotein that mediates cell-to-cell and cell-to-matrix interactions, plays a role in platelet aggregation, modulates arterial thrombosis in conjunction with von Willebrand factor, and contributes to vaso-occlusive complications, mucosal damage and pulmonary vascular remodeling and vasoconstriction.[15] CXCL2 is a chemokine that contributes to the inflammatory activation of vascular endothelial cells. Epiregulin is a ligand for the epidermal growth factor receptor that contributes to inflammation, angiogenesis and vascular remodeling.

We were able to measure plasma thrombospondin-1 concentration in 95 control and 110 CP individuals with the Human Thrombospondin-1 Quantikine Elisa Kit (R&D Systems, USA). In keeping with increased *THBS-1* expression in PBMC, reticulocyte and granulocyte peripheral blood fractions from patients with CP, we observed that plasma thrombospondin-1 concentrations were higher in patients than in controls (median 3448 ng/ml *versus* 2434 ng/ml, respectively; Figure 1D). We then examined the relationship of plasma thrombospondin-1 concentration with new thrombosis in 110 patients with CP, and observed an interaction of thrombospondin-1 concentration with the number of past thromboses on the odds of new thrombosis (*P*=0.034). We therefore examined the rate of new thrombosis in a subset of 101 CP patients who had a past history of no thrombosis or only 1 thrombosis. The occurrence of new thrombosis was higher with a plasma thrombspondin-1 concentration above the median of 3448 ng/ml. The crude hazards ratio was 1.5, 95% CI 0.6-3.6, *P*=0.39. In an analysis that controlled for age, treatment with pentoxifylline, phlebotomy category and smoking history; factors that were identified above; the hazards ratio was 2.8, 95% CI 1.05-7.4, *P*=0.034; Figure 1E.

There are a number of limitations to our study. We cannot rule out a possibility that those patients with previous thromboses and those perceived to be at higher thrombotic risk were more likely to be treated with pentoxifylline or phlebotomy, and that the observed relationships with new thrombosis may have been due to indication bias. Nevertheless, our findings raise the possibility that deregulation of the HIF pathway contributes to the elevated rate of thrombosis in patients with CP, and that genes such as *THBS1*, *CXCL2* and *EREG* are part of this process. Further studies are needed to elucidate the cell type-specific induction of these genes and their role in thrombosis. Such investigations may identify new pathways of prevention and treatment with broad applicability. More detailed evaluation of the risks and benefits of phlebotomy therapy in congenital and acquired polycythemias and myeloproliferative neoplasms may also be in order.

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