# The phenotype of polycythemia due to Croatian homozygous *VHL* (571C>G:H191D) mutation is different from that of Chuvash polycythemia (*VHL* 598C>T:R200W)

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## ABSTRACT

Mutations of *VHL* (a negative regulator of hypoxia-inducible factors) have position-dependent distinct cancer phenotypes. Only two known inherited homozygous *VHL* mutations exist and they cause polycythemia: Chuvash R200W and Croatian H191D. We report a second polycythemic Croatian H191D homozygote distantly related to the first propositus. Three generations of both families were genotyped for analysis of shared ancestry. Biochemical and molecular tests were performed to better define their phenotypes, with an emphasis on a comparison with Chuvash polycythemia. The *VHL* H191D mutation did not segregate in the family defined by the known common ancestors of the two subjects, suggesting a high prevalence in Croatians, but haplotype analysis indicated an undocumented common ancestor ~six generations ago as the founder of this mutation. We show that erythropoietin levels in homozygous *VHL* H191D individuals are higher than in *VHL* R200W patients of similar ages, and their native erythroid progenitors, unlike Chuvash R200W, are not hypersensitive to erythropoietin. This observation contrasts with a report suggesting that polycythemia in *VHL* R200W and H191D homozygotes is due to the loss of JAK2 regulation from *VHL* R200W and H191D binding to SOCS1. In conclusion, our studies further define the hematologic phenotype of *VHL* H191D and provide additional evidence for phenotypic heterogeneity associated with the positional effects of *VHL* mutations.

## Introduction

Von Hippel-Lindau (*VHL*) is a tumor-suppressor gene, mutations of which have long been recognized to predispose to renal cancer, pheochromocytoma and other cancers.[1] Notably, mutations clustering at different positions appear to favor certain tumor types.[2-4] VHL is a negative regulator of hypoxia inducible transcription factors (HIF). Two mutations located at the 3' portion of the *VHL* coding region cause polycythemia but not VHL syndrome cancers. The most frequent cause of congenital polycythemia is homozygosity for the hypomorphic *VHL* 598C>T (R200W) mutation leading to a reduced rate of ubiquitination of α subunits of HIF-1 and HIF-2, the principal mechanism underlying Chuvash polycythemia.[5] This disease is endemic in the Chuvash Autonomous Republic of the Russia Federation[6] and in the Italian island of Ischia,[7] and is sporadic worldwide; it is associated with decreased survival of homozygotes partly due to cerebral vascular events and systemic thrombosis. Other common manifestations are varicose veins and vertebral hemangiomas.[8] Haplotype analyses demonstrated that the mutation likely arose from a single founder 14,000 – 62,000

years ago, indicating a survival advantage of heterozygotes,[9] which may in part be due to protection from anemia.[10] In addition, compound heterozygosity for R200W and other *VHL* mutations has been reported in a few patients with congenital polycythemia.[11-14]

HIF are master transcription factors that determine cellular responses by oxygen-dependent destruction of α subunits. VHL is a substrate-recognition component of an E3 ubiquitin-protein ligase complex that, under normoxic conditions, ubiquitinates HIF1α and HIF2α and targets them for proteasomal degradation.[15] Disruption of the interaction between the α-subunits of HIF and VHL protein causes accumulation of HIF and altered transcription of downstream target genes including those for glucose transporter-1 (*SLC2A1*), vascular endothelial growth factor (*VEGF*), transferrin (*TF*) and erythropoietin (*EPO*).[5]

Eight years ago we described the first example of a homozygous germ-line *VHL* mutation other than the *VHL* R200W mutation, i.e. the 571C>G (H191D) mutation in a polycythemic boy from a region in the south of Croatia called Dalmatia.[16] This Croatian *VHL* mutation is positioned in the same structural region as the Chuvash polycythemia muta-

tion that leads to a modest partial loss of VHL activity since the affected residue is distant from the functional VHL domain.[17]

A high level of erythropoietin, due to increased HIF, was assumed to be a major cause of polycythemia in patients with these *VHL* R200W and H191D mutations, which are hallmarks of secondary polycythemic disorders. However, Chuvash polycythemia R200W erythroid progenitors are also hypersensitive to erythropoietin,[5,18] a hallmark of primary polycythemic disorders, by an as of yet unknown underlying mechanism. Recently, Russell and colleagues hypothesized that the mutated VHL R200W and H191D regions bind more avidly to suppressor of cytokine signaling 1 (SOCS1), a potent negative regulator of erythropoiesis.[19] They proposed that this abnormal association between the VHL protein and SOCS1 hinders Janus kinase 2 (JAK2) degradation leading to JAK2 up-regulation, which can potentially explain the erythroid hypersensitivity to erythropoietin observed in Chuvash polycythemia, and they predicted that this erythroid hypersensitivity to erythropoietin would also be observed in the Croatian *VHL* H191D mutation.[19]

In this study, we report another homozygous patient for the *VHL* 571C>G (H191D) germ-line mutation, a 5-year old Croatian girl from Herzegovina, a region located in the southern part of Bosnia contiguous to Dalmatia. Herzegovina is the counterpart to the Croatian region and is populated largely by people of Croatian ethnicity along the border. We set out to define the phenotype of this mutation in this and the previously reported homozygote[16] with particular emphasis on a critical comparison with Chuvash polycythemia. We also pursued a hypothesis that a putative survival advantage of H191D heterozygotes may account for the possible high prevalence of heterozygosity in Croatians and thus set out to determine its approximate origin in evolution by determining haplotype sharing among affected individuals.

## Design and Methods

Blood samples from 23 persons were collected from two different families, including two propositi with polycythemia and 21 relatives (Figure 1). Peripheral blood samples were collected into tubes containing EDTA and/or ACD. Written informed consent was obtained from all participants. The Institutional Review Board of the University of Utah approved the study.

### Mutation analysis of the *VHL* gene

Genomic DNA was isolated from granulocytes using the TRI reagent (Molecular Research Center, Cincinnati, OH, USA). For genotyping, 100 ng of patients' genomic DNA was used. Polymerase chain reactions (PCR) were performed using the HotStar Taq Master Mix Kit (QIAGEN, Germantown, MD, USA) and the following primers: VHL F agttgttggcaaagcctctt, VHL R caaaagctgagatgaaacagtg. As the *MslI* endonuclease abolishes a restriction site of the *VHL* 571C>G mutation, the PCR product was then purified with a QIAquick PCR Purification Kit (QIAGEN) and subjected to restriction with *MslI* enzyme (New England Biolabs, Beverly, MA, USA) according to the manufacturers' instructions. Cleavage products were evaluated on 2% agarose gels. PCR-direct sequencing was performed to confirm the mutation screening, using a standard protocol and the same amplification primers.

### Analysis of recent shared ancestry

The two individuals homozygous for *VHL* H191D were genotyped using the Illumina HumanOmni1-Quad BeadChip at the Children's Hospital of Philadelphia, Center for Applied Genomics. Beagle 3.2[20] was used to phase and impute missing genotypes, with the phase two release of 30 HapMap CEU trios as a reference.[21] GERMLINE 1.4.1[22] inferred the locations and extents of identity by descent (IBD) segments (parameters err_het = 2, err_hom = 1, and min_m = 1cM, with marker positions given on the HapMap r22 genetic map). ERSA 1.0 was applied to the GERMLINE output to test for evidence of recent shared ancestry.[23]
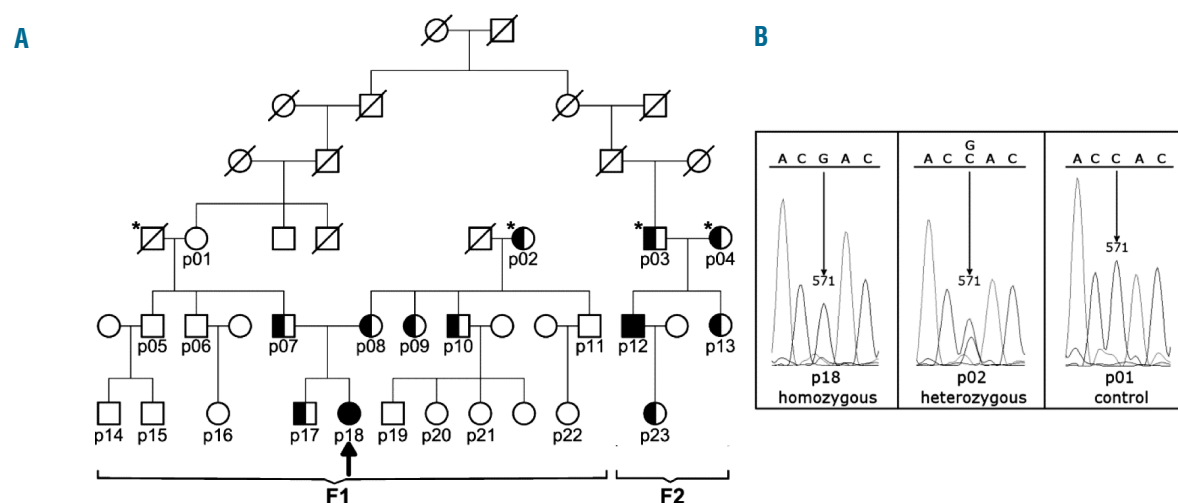


Figure 1. Pedigree with genotyping. (A) Pedigree of the family available for study with extended family history. Each family is denoted by a family number (F1, F2). The "p" code (p01, p02 and so on) indicates individuals from whom DNA samples have been obtained. The arrow indicates a new polycythemic patient. The *VHL* mutation of the paternal side of F1 must have been inherited from the husband of p01. The husband of p01 was not related to F2 according to the pedigree and the mutation did not segregate among the known relatives. Heterozygote individuals denoted by an asterisk as well as the husband of p01 presumably shared the common ancestor, the founder of the mutation. (B) Examples of sequences of the third exon of the *VHL* gene in an unaffected subject (p01), in a heterozygote subject (p02), and in a homozygote (p18) patient.

### In vitro *assay of the sensitivity of erythroid progenitors to erythropoietin*

*In vitro* sensitivity of erythroid progenitors to erythropoietin was determined on mononuclear cells isolated from the peripheral blood using Histopaque (Sigma-Aldrich, St. Louis, MO, USA) density gradient centrifugation and plating ($2.3 \times 10^5$/mL) on methylcellulose media (MethoCult® H4531; StemCell Technologies, Vancouver, BC, Canada) without addition of erythropoietin or with addition of various concentrations of erythropoietin (StemCell Technologies), ranging from 0.015 to 3.0 U/mL. Cell cultures were maintained in a humidified atmosphere of 5% $CO_2$ at 37°C for 14 days. Erythroid burst-forming unit colonies (BFU-E) were scored by standard morphological criteria. The assay was carried out on erythroid progenitors from one homozygous and three heterozygous *VHL* H191D individuals, two wild-type relatives and a polycythemia vera patient as a positive control. The protocol and interpretation of these assays were identical to those used for homozygous and heterozygous native progenitors with the Chuvash polycythemia *VHL* R200W mutation.[5]

### In vitro *expansion of human erythroid progenitors in liquid culture*

The progenitor cells were expanded from the mononuclear cell population using our published protocol.[24] Briefly, $1 \times 10^6$ cells/mL were cultured in StemSpan™ Serum-Free Expansion Medium (StemCell Technologies) containing different cytokine cocktails from day 1-7 (100 ng/mL of fetal liver tyrosine kinase 3 ligand, 100 ng/mL of thrombopoietin, and 100 ng/mL of stem cell factor), day 8-14 (50 ng/mL of stem cell factor, 50 ng/mL of insulin-like growth factor-1, and 3 U/mL of erythropoietin), and day 15-21 (50 ng/mL of insulin-like growth factor-1, and 3 U/mL of erythropoietin). All cytokines were a kind gift from Amgen (Thousand Oaks, CA, USA).

### *Real-time polymerase chain reaction assay*

Total RNA was isolated from granulocytes using TRI reagent solution (Molecular Research Center, Cincinnati, OH, USA) and then treated with DNA-free™ DNase Treatment & Removal Reagents (Ambion, Life Technologies, NY, USA) to remove any contaminating DNA. Five hundred nanograms of DNA-free RNA were reverse-transcribed using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Life Technologies, NY, USA) according to the manufacturer's instruction protocol. Quantitative PCR were performed with specific TaqMan® Gene Expression probes (Applied Biosystems, Carlsbad, CA, USA) for the following genes: *ADM* (Hs00181605), *TFRC* (Hs00951083), *NDRG1* (Hs00608387), *PDK1* (Hs00176853), *SLC2A1* (Hs00892681), *VEGF* (Hs00900055), *BNIP3* (Hs00969291), *BNIP3L* (Hs00188949), and *HK1* (Hs00175976). All samples were assayed in triplicate. Data were normalized to *HPRT* (4333768F) and *GAPDH* (4333764F) reference genes. The statistical significance of relative expression changes of target mRNA levels normalized to a reference genes was analyzed by the pair-wise fixed reallocation randomization test using REST© 2009 software.[25]

### *Biochemical studies*

The complete blood count was performed by an automated analyzer (Sysmex XT 2000i, Sysmex Corporation, Kobe, Hyogo, Japan). The concentration of erythropoietin in the serum was determined by an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA).

### *Statistical analysis*

Analyses were performed with Stata 10.1 (StatCorp., College Station, TX, USA).

## Results

### *Patients' characteristics*

The proposita is a 5-year old girl who was referred to the University Hospital of Zagreb due to failure to thrive at the age of 2 years. Routine blood analysis at the time of the current study revealed an erythrocyte count of $8.42 \times 10^{12}$/L and hemoglobin concentration of 14.5 g/dL. She was iron deficient (ferritin 6.3 µmol/L) and her erythropoietin was increased (202 mIU/mL; normal range, 4-28). Her weight and height were below the 5th percentile for age and gender and psychomotor development was delayed, but she was otherwise normal. She experiences headaches about twice weekly, on average. Besides adequate hydration and daily acetylsalicylic acid (1 mg/kg), she receives no other treatment. Her parents were born in the same part of the country and deny being related.

The previously reported homozygous patient was diagnosed with polycythemia and elevated erythropoietin at the age of 12 years. He is now 26 years old and has occasional headaches and malaise, and tires easily. He is phlebotomized once or twice a month depending on symptoms and hematologic findings. He has been iron deficient because of the regular phlebotomies to reduce hemoglobin concentration.

We found no individuals among the heterozygotes who had evidence or a history of polycythemia. Neither homozygote had evidence of varicose veins which are common in young Chuvash polycythemia patients and virtually invariable after the age of 18 years.[8]

### *Analysis of the* VHL *H191D mutation and mapping of the* VHL *locus*

Among 23 members (12 female, 11 male) of the families of the two propositi, we found 10 heterozygotes (6 female, 4 male; Figure 1) for the *VHL* H191D mutation. The father of the proposita is distantly related to the first *VHL* H191D homozygote polycythemic subject but genotyping revealed that the *VHL* mutation in family F1 must have come from the husband of p01 who was not related to family F2 (Figure 1). Thus, the *VHL* H191D mutation does not segregate among the known relatives between these two families, and no evidence suggests that the mutation originated from one of two known common ancestors depicted in Figure 1A.

We analyzed the two homozygotes (p12 and p18) by high-density genotyping to examine their relatedness and the *VHL* haplotype. We detected evidence of a significant relationship between the two individuals ($P=4 \times 10^{-18}$), with a maximum likelihood estimate of 8th degree relatives (95% CI 6th degree - 11th degree)[23], exactly matching the known pedigree (Figure 1). These individuals share a 16 cM haploid segment on chromosome 11 and a 24 cM haploid IBD segment on chromosome 3 (identical copies of a gene segregating from a common ancestor within the defined pedigree, Figure 2). Within the segment on chromosome 3, a 15.6 cM autozygous segment is present in the female (p18), and a 1.6 cM autozygous segment is present in the male (p12). Both autozygous segments contain the *VHL* mutation, and the smaller segment is diploid IBD (Figure 2). Two other large autozygous segments are present in the male (15.5 cM on chromosome 15 and 10 cM on chromosome 4). The presence of autozygosity at the *VHL* mutation and elsewhere in the genome is a strong indication that all four of the parents of p12 and

p18 are related[23] and share a common ancestor. This common ancestor is likely the founder of the *VHL* H191D mutation. Because *VHL* H191D does not segregate among the known relatives of the two families, the evidence for a common founder indicates multiple undocumented relationships. The four carriers of this mutation in the generation of the grandparents are p02, p03, p04, and the husband of p01 (Figure 1). The size and number of autozygous (two alleles at a locus originate from a common ancestor by way of non-random mating) segments in p12

and p18 suggest that the four carriers in the grandparental generation are separated from the common founder by two to five generations (by 3-6 generations for propositi).

### Response of erythroid progenitors to erythropoietin

We performed the erythroid progenitor colony (BFU-E) assay on peripheral blood mononuclear cells from one *VHL* H191D polycythemic patient, heterozygous subjects and two healthy controls. The growth of BFU-E of the affected individuals and mutation carriers, unlike those of
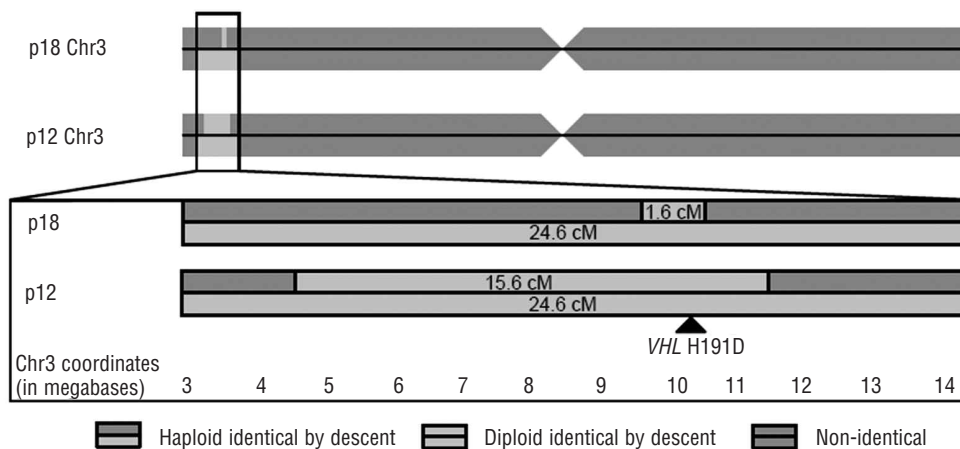


**Figure 2.** IBD (gene identity by descent) and autozygosity on chromosome 3. The two individuals homozygous for *VHL* H191D share a 24.6 cM haploid IBD segment, a 15.6 cM haploid IBD segment that is autozygous in the female, and a 1.6 cM diploid IBD segment that is autozygous in both individuals. All segments contain the *VHL* mutation. The presence of large autozygous IBD segments strongly suggests that all four copies of the *VHL* mutation originated from a recent founder.
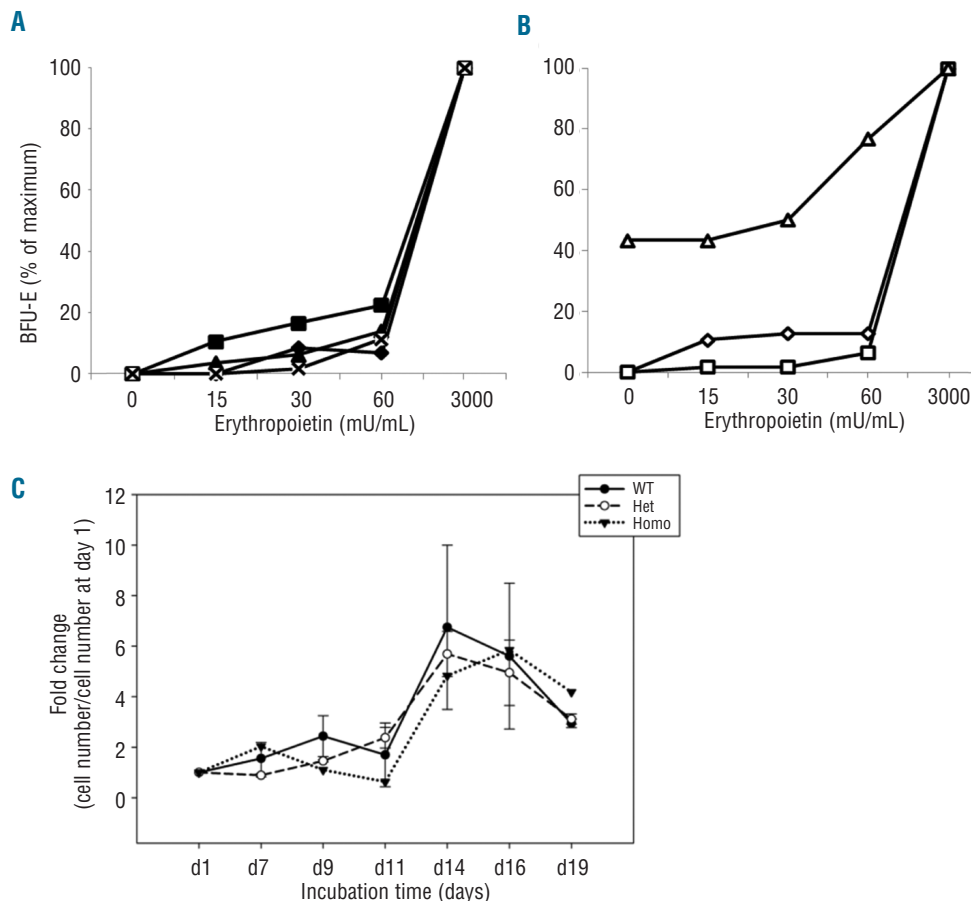


**Figure 3.** Sensitivity to EPO and *in vitro* proliferation of BFU-E erythroid progenitors. (A) Erythroid progenitor growth curves show the relative percentages of colonies, i.e. the percentage of colonies of each genotype, for a given concentration of erythropoietin, relative to that at the maximum concentration (3000 mU/mL). Normal response of BFU-E to erythropoietin was found in the patient homozygous for *VHL* H191D mutation (p12,×). Heterozygous individuals (p03, ◆), (p04, ■), (p08, ▲) also showed normal responses in the presence of a low concentration of erythropoietin. (B) Concomitantly tested healthy controls (◇), (□) and a patient with polycythemia vera used as a hypersensitive control (△). (C) *In vitro* expansion of cells from homozygous *VHL* H191D patient (p12), three heterozygous individuals (p03, p04 and p08) and healthy controls (n=2). The number of expanded cells was determined at each time point. Fold increase in the number of cells was determined from the initial total number of peripheral blood progenitors used for expansion. Data are presented as the mean and the whiskers represent SE intervals.

Chuvash polycythemia homozygotes, resembled the growth of normal controls (Figure 3A,B). The result of the second homozygous *VHL* H191D patient (p18) is not included in Figure 3A because only a limited amount of her peripheral blood progenitors was available, precluding the whole erythropoietin response curve. However, no BFU-E colonies were observed at low erythropoietin concentrations (15, 30 and 60 mU erythropoietin/mL). Concomitantly analyzed BFU-E from a patient with polycythemia vera were markedly hypersensitive to erythropoietin. Proliferation of *VHL* H191D erythroid progenitors in liquid cultures was also normal, when compared to that of healthy controls (Figure 3C).

### Effect of the VHL *H191D mutation on expression of the target genes of hypoxia inducible factors 1 and/or 2*

We evaluated the expression level of several target genes of HIF-1 and/or HIF-2 (*ADM, TFRC, NDRG1, PDK1, SLC2A1, VEGF, BNIP3, BNIP3L* and *HK1*) in granulocytes from the two homozygous patients and eight normal controls (Figure 4). The homozygotes for *VHL* H191D mutation had increased expression of several HIF-1 and/or HIF-2 regulated genes (*TFRC, SLC2A1, VEGF, BNIP3* and *HK1*) and two genes (*ADM* and *BNIP3L*) were down-regulated. Changes of expression of HIF target genes are often tissue-specific and our analyses were limited to only available tissue to which the studied subjects consented.

### Comparison of clinical variables and biological markers among VHL *genotypes*

The clinical characteristics of the study participants are summarized in Table 1 according to *VHL* phenotype. Comparing *VHL* H191D heterozygotes with subjects with wild-type *VHL*, it was found that the former had higher mean values of the mean corpuscular volume (*P*=0.030) and of serum ferritin concentration (*P*=0.007) and lower mean cell hemoglobin concentration (*P*=0.033). The limited number of homozygous samples precludes their statistical evaluation, and furthermore the clinical studies in the homozygotes are affected by the fact that one of them has been frequently phlebotomized. Consistent with the phlebotomy program of p12 and the iron deficiency of both homozygotes (p12, p18), mean corpuscular volume, mean cell hemoglobin, mean cell hemoglobin concentration and ferritin were decreased among the *VHL* H191D homozygotes compared to both the other two groups: *VHL* wild-type and *VHL* H191D heterozygotes. Nevertheless, the hematocrit and red blood cell count were increased among the *VHL* H191D homozygotes. The extremely low mean cell hemoglobin and mean cell hemoglobin concentration could explain the discrepancy between red blood cell volume hematocrit and hemoglobin concentration in addition to the high concentration of erythropoietin, which was increased despite the lack of anemia. Moreover, high erythropoietin is associated with plasma volume contraction[26] which can further elevate hematocrit. The erythropoietin concentrations of both *VHL* H191D homozygotes are higher than those of Chuvash polycythemia patients of similar age and with similar hemoglobin concentration (Table 1 and Figure 5). We also compared other hematologic parameters between *VHL* H191D homozygotes and heterozygotes and relevant Chuvash polycythemia counterparts (Table 1). The *VHL* R200W heterozygotes were older and had lower mean corpuscular volumes and ferritin levels compared to the *VHL* H191D heterozygotes.

## Discussion

We describe a 5-year old Croatian girl with recessively inherited congenital polycythemia due to homozygosity for the *VHL* H191D mutation. We studied her extended family, as well as revisited and expanded the study of the originally described polycythemic patient with the same *VHL* H191D mutation[16] and also his extended family. This homozygous *VHL* mutation, along with Chuvash polycythemia *VHL* R200W, are the only *VHL* mutations thus far not predisposing to tumor development but rather causing polycythemia.

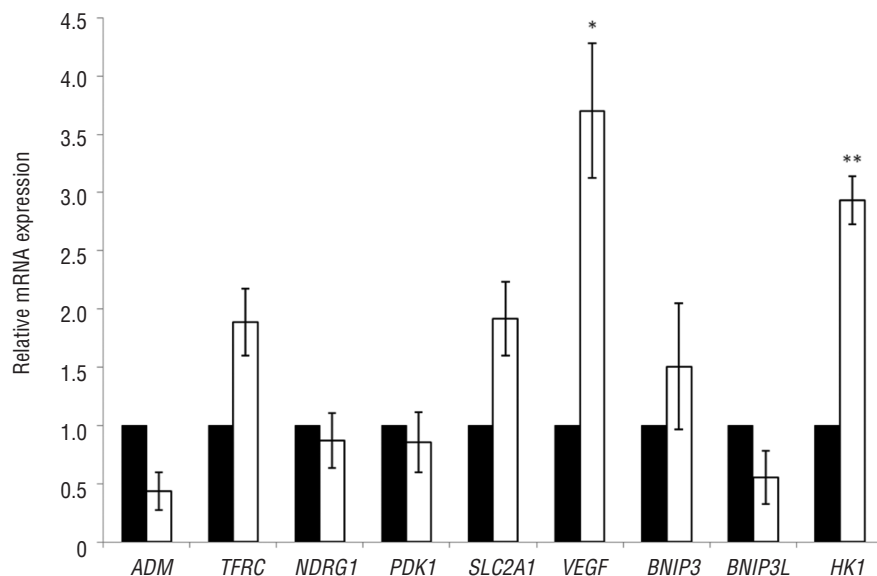While we had previously shown[5] that in Chuvash poly-



**Figure 4.** Expression of HIF-1 and HIF-2 target genes. Expression of *ADM, TFRC, NDRG1, PDK1, SLC2A1, VEGF, BNIP3, BNIP3L* and *HK1* genes was evaluated by quantitative PCR in granulocytes isolated from two patients homozygous for *VHL* H191D mutation (p12, p18, white columns) and normal controls (n = 8, black columns). Data are normalized to *HPRT* and *GAPDH* reference genes. All samples were investigated in triplicate. Results pooled from three separate experiments are shown. The bars represent mean mRNA levels relative to the mean levels calculated for wild-type controls. The whiskers represent SE intervals. Significant mean value deviations from normal controls are presented as *(*P*<0.05) and **(*P*<0.01).

cythemia, the erythroid progenitors are intrinsically hyperproliferative (a feature of primary polycythemia), their average elevated erythropoietin levels (a feature of secondary polycythemia) further contribute to augmented erythropoiesis. No differences of BFU-E response of Chuvash heterozygotes have been detected in our multiple studies over the last decade. The *VHL* H191D mutation differs from *VHL* R200W, as the erythropoietin levels seen in both *VHL* H191D homozygotes appear to be higher than those in patients with Chuvash polycythemia, but more importantly, we have been unable to find evidence of a primary polycythemic functional defect in the native erythroid progenitors of this family. We can, therefore, conclude that the *VHL* H191D polycythemic phenotype is solely driven by erythropoietin.

There is a significant, non-erythroid phenotype in Chuvash polycythemia; specifically, there is an increased risk of thrombotic and hemorrhagic strokes, non-central nervous system thrombotic complications, pulmonary hypertension, and other abnormalities[8] which are associated with decreased survival of homozygotes and, thus, one would expect a negative survival pressure of the *VHL* R200W mutation. However, examination of the population effect of this mutation in a large number of individuals of Asian-Indian, Caucasian and Chuvash (population from Central Asia) origin suggested that this mutation arose from a single founder, possibly prior to diversification of the human races.[9] This implies that there is some, possibly subtle survival advantage for heterozygotes which allows this mutation not only to persist, but to increase. As shown in Figure 1, the *VHL* H191D mutation in two seemingly unrelated individuals led us to examine whether this mutation may be much more frequent in

Croatians, perhaps exhibiting a similar survival advantage for heterozygotes for *VHL* H191D as that shown for *VHL* R200W. However, our haplotype analysis demonstrated a recent origin of this mutation and, thus, the frequency of this mutation cannot be used in arguments for or against any survival benefit or detriment.

The increased intrinsic sensitivity to erythropoietin of erythroid progenitors with the *VHL* R200W mutation has not yet been clarified. A recent paper[19] suggested that this is due to selective binding of the mutated VHL protein in the regions of *VHL* H191D and *VHL* R200W to an inhibitor of erythropoiesis (SOCS 1). However, this report erroneously quoted the *VHL* H191D mutation as a Chuvash polycythemia mutation and concluded that the same erythropoiesis-augmenting mechanism applies for both mutations. Clearly, this is not the case, as shown by our data demonstrating no intrinsic augmented erythroid proliferation with *VHL* H191D, unlike that present in erythroid progenitors bearing the *VHL* R200W mutation. By repeated testing of many Chuvash and non-Chuvash individuals homozygous for the *VHL* R200W mutation, we consistently showed that BFU-E from patients with Chuvash polycythemia are erythropoietin hypersensitive. Unfortunately, we did not have enough material, or consent from the patients with *VHL* H191D for repeated blood sampling, which precluded SOCS 1 analysis in their BFU-E.

We evaluated hematologic and iron data from our heterozygous and two homozygous patients and compared them to our previous findings in homozygous and heterozygous relatives of patients with Chuvash polycythemia. The *VHL* H191D heterozygotes had larger mean cell volumes and ferritin concentrations than the

**Table 1.** Comparison of clinical variables between individuals with *VHL* H191D or *VHL* R200W mutations.

| | *VHL* wildtype, *VHL* H191D and *VHL* R200W heterozygotes[#] | | | | | *VHL* H191D and *VHL* R200W homozygotes[§] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *VHL* wildtype (n = 11) | *VHL* H191D heterozygote (n = 10) | $P^\delta$ | *VHL* R200W heterozygote (n = 34)[10] | $P^\&$ | p18 | *VHL* R200W homozygote comparison for p18 (n =1) | p12 | *VHL* R200W homozygote comparisons for p12 (n = 20; results in range) |
| Age (years) | 26±2 | 35±2 | 0.3 | 53±2 | <0.001 | 5 | 16 | 26 | 25-56 |
| N. of females | 5 (45%) | 6 (60%) | 0.5 | 19 (56%) | NS | female | male | male | 13 (65%) |
| HBF (%) | 1.3±0.4 | 1.5±0.4 | 0.4 | ND | | 0.5 | ND | 1.4 | ND |
| Hemoglobin (g/dL)* | 14.0±0.3 | 14.1±0.3 | 0.8 | 13.4±0.2 | 0.09 | 14.50 | 16.4 | 13.7 | 12.2-15.7 |
| Hematocrit (%)* | 44.6±1.0 | 46.1±1.0 | 0.3 | ND | | 53.1 | 53.4 | 59.6 | 37.8-51.8 |
| RBC*x10[6] (cells/µL) | 4.94±0.08 | 4.78±0.08 | 0.2 | ND | | 8.42 | 6.58 | 8.89 | 4.59-7.59 |
| MCV (fL)** | 89.9±1.8 | 96.8±1.9 | 0.030 | 88±0.8 | <0.0001 | 63.1 | 81.2 | 67.1 | 59.6-93.3 |
| MCH (pg)** | 28.4±0.6 | 29.7±0.6 | 0.172 | ND | | 17.2 | 24.9 | 15.4 | 18.3-32.7 |
| MCHC (g/dL)** | 31.5±0.2 | 30.6±0.3 | 0.033 | ND | | 27.3 | 30.7 | 23.0 | 30.3-35.4 |
| WBCx10[3] (cell/µL) ** | 5.6±0.52 | 6.46±0.55 | 0.3 | 7.6±0.29 | 0.1 | 6.4 | 5.96 | 4.1 | 3.5-8.8 |
| Plateletsx10[3] (cell/µL) ** | 260±25 | 290±26 | 0.4 | 238±12 | 0.053 | 310 | 247 | 199 | 114-369 |
| Ferritin (ng/mL)[§]* | 39 (32-48) | 112 (88-143) | 0.007 | 62 (57-73) | 0.005 | 6.3 | 11.9 | 2.3 | 0.4-20.0 |
| Erythropoietin (mIU/mL)*** | 8.2 (6.1-10.7) | 9.6 (7.2-26) | 0.7 | 11 (10.8-11.2) | 0.4 | 201.6 | 31 | 469 | 27-230 |

*[#]Results as mean ± SE or mean (range), unless otherwise indicated. [§]VHL R200W homozygote comparisons for either pediatric or adults subjects with hemoglobin concentration within 2 g/dL of the Croatian subjects. [δ]Comparison of VHL wildtype and VHL H191D heterozygotes. [&]Comparison of VHL H191D and R200W heterozygotes. [§]Geometric mean and SE range. For analysis of ferritin, two outliers excluded. *Analysis by ANOVA with adjustment for pedigree and gender. ** Analysis by ANOVA with adjustment for pedigree. *** Analysis by ANOVA with adjustment for pedigree and hemoglobin; geometric mean and SE range. RBC: red blood cells; MCV: mean corpuscular volume; MCH: mean cell hemoglobin; MCHC: mean cell hemoglobin concentration; WBC:white blood cells.*
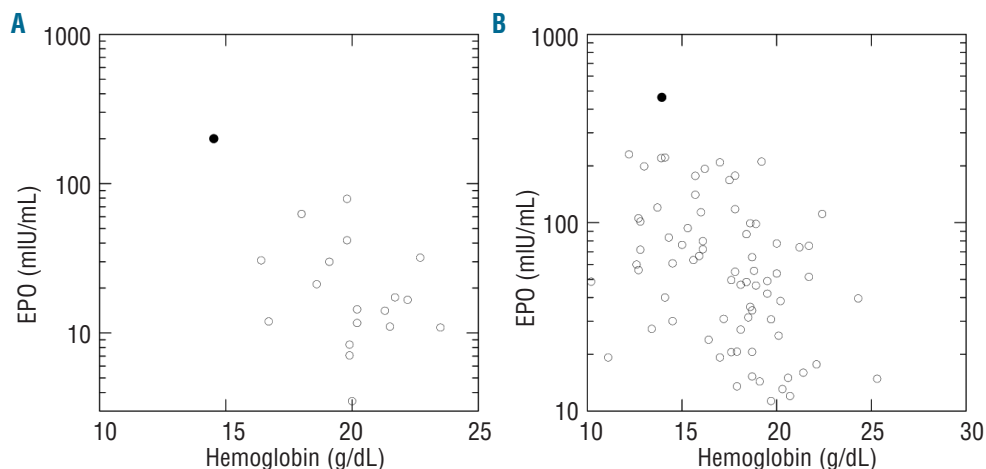
**Figure 5.** Serum EPO levels in VHL H191D Croatian patients, as compared to CP patients of similar age. The vertical axes represent logarithmic scale of serum EPO values in relation to hemoglobin concentration on horizontal axes. (A) Croatian p18 patient, and CP patients up to 20 years old (B) Croatian p12 patient, and CP patients at least 20 years old. EPO concentration is higher in Croatian (●) than in CP patients (○).

R200W heterozygotes but similar hemoglobin and erythropoietin concentrations.[10] The two *VHL* H191D homozygotes tended to have higher erythropoietin concentrations relative to the hemoglobin concentration compared to *VHL* R200W homozygotes.[8] In both types of polycythemia (Chuvash and Croatian) our data indicate an impaired interaction of VHL with hypoxia inducible factors, reducing the degradation rate of alpha subunits resulting in increased expression of downstream target genes including *SLC2A1*, *TFRC* and *VEGF*.

In summary, our data provide additional evidence of an as yet unexplained variations of phenotypes with different locations within the *VHL* gene. While *VHL* is a relatively large gene (12 kb) with three exons and two introns, the coding region consists of only 213 codons. The resultant very small protein has been well-studied as a cause of one of the first and most comprehensively investigated tumor predisposition syndromes. Germ-line mutations predicted to cause truncated protein and exon deletions are characteristic for the development of retinal and central nervous system hemangioblastomas and clear renal cell carcinoma, while germ-line missense mutations typically cause pheochromocytomas.[27] Thus, it was unexpected that the large number of patients with Chuvash polycythemia (bearing the *VHL* R200W mutation either in heterozygous or homozygous forms) have not been associated with any classical VHL syndrome tumors, but with polycythemia. We now provide some evidence that patients with the *VHL* H191D mutation, which is positionally close to the *VHL* R200W mutation, appear to have a similar, but apparently not identical, phenotype suggesting subtle functional differences of these two different *VHL* mutations. More work is needed to define the molecular basis of the array of phenotypic differences arising from changes in this rather small VHL peptide.

### *Authorship and Disclosures*

*Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.*

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