Published in final edited form as:

Br J Haematol. 2013 September ; 162(6): 851–853. doi:10.1111/bjh.12431.

Novel Compound VHL Heterozygosity (*VHL* T124A/L188V) Associated With Congenital Polycythaemia

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Keywords

polycythaemia; erythrocytosis; mutations; molecular haematology; genetics

Familial polycythaemia (erythrocytosis) is comprised of several inherited disorders arising from diverse aetiological factors. Primary polycythaemias are characterized by intrinsic defects within erythroid progenitors, which either grow independently or are hypersensitive to erythropoietin (EPO), while secondary polycythaemias are from circulating factors, such as EPO. Secondary familial polycythaemias are due to increased haemoglobin oxygen affinity (α , β , and γ globins and bisphosphoglycerate mutase mutations), while the primary familial polycythaemias are due to gain-of-function of EPO receptor (Gordeuk, *et al* 2005) and T-cell acute leukaemia 1 (TAL1) (Rogers, *et al* 2012) mutations. Mutations of the hypoxia-inducible factor (HIF) pathway present as either secondary (Tomasic, *et al* 2013) or with features of both primary and secondary polycythaemias (Chuvash polycythaemia) (Gordeuk, *et al* 2005). HIF pathway mutations include gain-of-function mutations of the *EPAS1* gene (also known as *HIF2A*) and loss-of-function mutations of two negative regulators of HIFs, *EGLN1* (also known as *PHD2*) and von Hippel-Lindau (*VHL*) genes (Gordeuk, *et al* 2005). *VHL*, *EGLN1* and *EPAS1* mutations have been reported to be associated with either familial polycythaemia or pheochromocytoma/paraganglioma, but rarely both (Ladroue, *et al* 2008, Lorenzo, *et al* 2013). Two homozygous mutations of the *VHL* gene, Chuvash (*VHL*^{R200W}) and Croatian (*VHL*^{H191D}) polycythaemias, both in exon 3, have been reported to cause polycythaemia but not tumours (Ang, *et al* 2002, Tomasic, *et al* 2013).

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Author contributions

FRL designed and performed research, analysed the data and drafted the paper. CY performed research, analysed data and reviewed the paper. LL performed the research, analysed data and reviewed the paper. LB contributed the subjects for study, reviewed and critically revised the paper. ZZ revised and critically reviewed the paper. JTP design the research, critically reviewed and revised the paper, and approved the final version for submission.

Conflict of interest

The authors have no financial or competing interests to disclose.

We now report a 15-year-old male and his 10-year-old sister of mixed European descent, polycythaemic since infancy, with elevated EPO, who have been intermittently phlebotomized since their diagnosis. The parents are haematologically normal and not known to be related, with no history of VHL tumours among extended family members. Their peripheral blood was collected, with Institutional Review Board approved informed consents signed by both parents, and studied.

Mutational screening of the HIF pathway genes coding region at *HIF1A*, *EPAS1* and *EGLN1* were negative for any mutation. However, we found two missense mutations in the *VHL* genes from the two probands. A novel heterozygous mutation in exon 2 NM_000551 c.370C>A (*VHL*: T124A) was found in the father and a previously reported pathogenic allele variant NM_000551 c.562C>G (*VHL*: L188V) (Ohh, *et al* 2000) in exon 3 in the mother; both probands were compound heterozygotes for these changes.

Erythroid progenitors from the probands and the father, but not the mother, were hypersensitive to EPO by peripheral blood burst-forming-unit-erythroid (BFU-E) colony assay (Fig. 1), a feature of primary polycythaemia (Ang, *et al* 2002, Gordeuk, *et al* 2005, Sokol, *et al* 1995). The relative expression of HIF target genes, *HK1*, *SLC2A1* and *TFRC*, from three biological replicate samples was significantly increased when compared to controls (data not shown).

We further investigated the downstream effects of protein expression and function on E3 ligase activity in HIF regulation by expressing recombinant wild-type and mutant VHL protein constructs in a VHL-deficient 786-0 renal cell adenocarcinoma cell line and assessed their interaction with HIF1A protein by Western blot. Both wild-type and mutant VHL bound HIF1A protein comparably (Fig. 2A). We then tested ubiquitination of the HIF1A protein and HIF1 bound and ubiquitinated by both tested VHL mutants as efficiently as wild type VHL (Fig. 2B). These results suggest that the intrinsic biological function of these two VHL mutants is intact. We then considered the possibility that these two mutants could have decreased stability. To test this, we conducted a cycloheximide assay to study protein folding, maturation, and degradation. The results showed that the stability of both VHL¹²⁴ and VHL¹⁸⁸ mutants were significantly reduced, with their half-lives decreased from ~8 h to ~2 h (Fig 2C,D).

We then generated a crystal structure model of HIF-VHL-ElonginB-ElonginC Complex. The predicted protein structure indicated that both amino acid residues lie in the interior pocket of the VHL protein and are less likely to involve physical contact with HIF (data not shown).

In summary, we describe a novel germline *VHL* exon 2 mutation present in the compound heterozygous state with an exon 3 *VHL* variant in two polycythaemic siblings. We showed that this compound heterozygosity of *VHL* is associated with elevated EPO (secondary polycythaemia) and also has characteristics of primary polycythaemia (EPO hypersensitivity). In addition, the heterozygous *VHL*^{T124A} mutation present in the father also exhibited BFU-E hypersensitivity. We then evaluated HIF activity by transcription of HIF-target genes. Granulocytes from compound *VHL*^{T124A, L188V} subjects had increased transcripts of the HIF-regulated genes *SLC2A1* and *TFRC*, which correlated with delayed HIF degradation due to a reduced half-life of VHL. It has been reported that *VHL* mutations accompanied by BFU-E EPO hypersensitivity are unique for distal mutations in *VHL* exon 3 (*VHL*^{H191D} and *VHL*^{R200W}) and are due to altered SOCS1 binding (a negative regulator of JAK2) (Russell, *et al* 2011). However, we also demonstrated BFU-E EPO hypersensitivity in these proximal exon 2 *VHL* mutations (Lanikova, *et al* 2013), indicating that other, yet to be defined molecular mechanism(s) of EPO-hypersensitivity must exist. We showed that the

loss of function of *VHL* mutations in this family with congenital polycythaemia is, at least in part, due to decreased stability of mutant VHL proteins resulting in delayed ubiquitination and degradation of HIF, and this increases HIF1A activity.

Acknowledgments

This work were supported by NIH-P01CA108671 and VA Merit Review Award (PI-Prchal). LL was in part supported by Czech Science Foundation (project P301/12/1503).

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Figure 1. Erythroid progenitor sensitivity assay of erythropoietin (EPO)

Burst-forming-unit-erythroid (BFU-E) colonies were enumerated and expressed as a percentage of maximum at stimulation with different EPO concentrations from each family member and controls.

Figure 2. VHL structural and functional studies

A–B: The efficient reduction of HIF1A by reintroduction of the VHL mutants into VHL-deficient 786-O cells in contrast with wild-type and empty vector. C–D: Cyclohexamide (CHX) chase assay for protein half-life shows that mutant VHL proteins have decreased half-lives. 786-O cells stably transfected with VHL variants and treated with CHX (200 μ M) for 4 h; hourly lysates were analysed by Western blot.