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Distinct deregulation of the hypoxia inducible factor by *PHD2* mutants identified in germline DNA of patients with polycythemia

Charline Ladroue,[1]* David Hoogewijs,[2]* Sophie Gad,[1] Romain Carcenac,[1] Federica Storti,[2] Michel Barrois,[3] Anne-Paule Gimenez-Roqueplo,[4] Michel Leporrier,[5] Nicole Casadevall,[6] Olivier Hermine,[7] Jean-Jacques Kiladjian,[8] André Baruchel,[9] Fadi Fakhoury,[10] Brigitte Bressac-de Paillerets,[3,11] Jean Feunteun,[12] Nathalie Mazure,[13] Jacques Pouysségur,[13] Roland H. Wenger,[2] Stéphane Richard,[1,14] and Betty Gardie[1]

[1]Génétique Oncologique EPHE, INSERM U753, Institut de Cancérologie Gustave Roussy, Villejuif et Faculté de Médecine Paris-Sud, Le Kremlin-Bicêtre, France; [2]Institute of Physiology and Zürich Center for Integrative Human Physiology ZIHP, University of Zürich, Zürich, Switzerland; [3]Service de Génétique, Institut de Cancérologie Gustave Roussy, Villejuif, France; [4]Assistance Publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou, Service de Génétique, Paris, France; INSERM, UMR970, Paris-Cardiovascular Research Center at HEGP, Paris, France; Université Paris Descartes, Faculté de Médecine, Paris, France; [5]CHU de Caen, Service d'Hématologie Clinique, France; [6]Hôpital Saint Antoine, Assistance Publique–Hôpitaux de Paris and Pierre et Marie Curie University, Institut Gustave Roussy, Inserm, UMR790, Villejuif, France; [7]Service d'Hématologie, Hôpital Necker-Enfants Malades, AP-HP, Paris, France; [8]Centre d'Investigations Cliniques, Hôpital Saint-Louis, AP-HP, Paris, France; [9]Service d'Hématologie, Hôpital St. Louis, Paris, France; [10]Service de Néphrologie, Hôpital Necker, Paris, France; [11]Unité INSERM U946, Variabilité Génétique et Maladies Humaines, Fondation Jean Dausset/CEPH Unité, Paris, France; [12]Institut de Cancérologie Gustave Roussy, CNRS-UMR8200, Villejuif, France; [13]Institut de Biologie du Développement et Cancer, Université de Nice - Sophia Antipolis, CNRS UMR-6543, France, and [14]Centre Expert National Cancers Rares INCa "PREDIR" and Réseau National INCa, AP-HP, Service d'Urologie, CHU, Le Kremlin-Bicêtre and Service de Néphrologie, Hôpital Necker, Paris, France

ABSTRACT

Background

Congenital secondary erythrocytoses are due to deregulation of hypoxia inducible factor resulting in overproduction of erythropoietin. The most common germline mutation identified in the hypoxia signaling pathway is the Arginine 200-Tryptophan mutant of the von Hippel-Lindau tumor suppressor gene, resulting in Chuvash polycythemia. This mutant displays a weak deficiency in hypoxia inducible factor α regulation and does not promote tumorigenesis. Other von Hippel-Lindau mutants with more deleterious effects are responsible for von Hippel-Lindau disease, which is characterized by the development of multiple tumors. Recently, a few mutations in gene for the prolyl hydroxylase domain 2 protein (*PHD2*) have been reported in cases of congenital erythrocytosis not associated with tumor formation with the exception of one patient with a recurrent extra-adrenal paraganglioma.

Design and Methods

Five *PHD2* variants, four of which were novel, were identified in patients with erythrocytosis. These *PHD2* variants were functionally analyzed and compared with the *PHD2* mutant previously identified in a patient with polycythemia and paraganglioma. The capacity of *PHD2* to regulate the activity, stability and hydroxylation of hypoxia inducible factor α was assessed using hypoxia-inducible reporter gene, one-hybrid and *in vitro* hydroxylation assays, respectively.

Results

This functional comparative study showed that two categories of *PHD2* mutants could be distinguished: one category with a weak deficiency in hypoxia inducible factor α regulation and a second one with a deleterious effect; the mutant implicated in tumor occurrence belongs to the second category.

Conclusions

As observed with germline von Hippel-Lindau mutations, there are functional differences between the *PHD2* mutants with regards to hypoxia inducible factor regulation. *PHD2* mutation carriers do, therefore, need careful medical follow-up, since some mutations must be considered as potential candidates for tumor predisposition.

Key words: hypoxia inducible factor, hypoxia-inducible transcription factor, *PHD2*, erythrocytosis.

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**Equal contribution to the work.*

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Correspondence:
Betty Gardie, Laboratoire de Génétique Oncologique EPHE, INSERM U753, Institut de Cancérologie Gustave Roussy, Villejuif, France.
E-mail: betty.gardie@inserm.fr

The online version of this article has a Supplementary Appendix.

Introduction

Secondary erythrocytosis is due to external factors such as increased production of erythropoietin, the origin of which is variable and may result from germline mutations in genes encoding factors involved in the oxygen-sensing pathway. The primary cellular component implicated in oxygen homeostasis is the hypoxia-inducible transcription factor (HIF). HIF operates as a heterodimer composed of a constitutively expressed beta subunit, also known as aryl hydrocarbon receptor nuclear translocator, and an alpha subunit (1α, 2α or 3α) that is tightly regulated by oxygen via post-translational modification. The prolyl-4-hydroxylase domain (PHD) enzymes hydroxylate proline residues located in the oxygen-dependent degradation (ODD) domain of HIF-α. This hydroxylation allows the binding of the von Hipple-Lindau protein (pVHL), the substrate recognition subunit of an E3 ubiquitin ligase complex that induces ubiquitination and subsequent degradation of HIF-α by the proteasome.[1,2] In the absence of oxygen, HIF-α is stabilized, heterodimerizes with HIF-1β and induces expression of hundreds of genes involved in cell survival, angiogenesis, erythropoiesis and cell proliferation.[2,3] There is some restricted target gene specificity depending on the HIF-α subunit of the HIF-α/β heterodimeric transcription factor. For example, renal and hepatic erythropoietin is regulated by the HIF-2α subunit *in vivo*.[4-7] Germline mutations in genes involved in the HIF pathway have been reported in association with syndromes that predispose patients to both neoplasms and/or congenital secondary erythrocytosis.[8] The most frequent mutations involve the *VHL* tumor suppressor gene. Heterozygous germline mutations in this gene are responsible for von Hippel-Lindau (VHL) disease, an autosomal dominant condition predisposing to multiple tumors including central nervous system and retinal hemangioblastomas, clear-cell renal cell carcinoma, pheochromocytomas and pancreatic endocrine tumors.[8] Established correlations between genotype and phenotype predict the risk of paraganglioma/pheochromocytoma, with *VHL* deletions or truncating mutations being associated with a low risk (VHL type 1) and *VHL* missense mutations being associated with a high risk (VHL type 2).[9,10]

In addition, a homozygous 598C>T (R200W) *VHL* germline mutation has been shown to account for Chuvash congenital polycythemia, an autosomal recessive disease, endemic in the Chuvash Autonomous Republic of the Russian Federation.[11] Homozygous carriers of the R200W-*VHL* germline mutation do not develop tumors but instead have Chuvash congenital polycythemia due to high levels of erythropoietin.[11] The lack of tumor development in this disorder is due to a weak defect of the mutation in terms of its HIF-α regulation (leading to delayed ubiquitination) because of its localization outside pVHL functional domains.[11] Other homozygous and compound heterozygous polycythemia-associated *VHL* mutations have also been reported.[10] Functional studies of some of these mutants have shown a weak to undetectable defect of HIF-1α regulation (*unpublished data*).

Recently, a similar phenotype of high erythropoietin-associated polycythemia without associated tumors has been reported in carriers of heterozygous germline mutations in the *PHD2* and *HIF-2A* genes,[12-19] with the exception of one patient carrying a H374R-*PHD2* mutation.[20] This particular patient simultaneously developed congenital secondary erythrocytosis and recurrent paraganglioma, a tumor originating from neural crest cells similar to pheochromocytoma but with an extra-adrenal localization.[20] The analysis of the tumor showed a loss of heterozygosity including the wild-type *PHD2* allele, suggesting a potential tumor suppressor role of *PHD2*.

PHD2 and VHL act in concert to regulate HIF-α. Based on the observation that *VHL* mutation carriers display different phenotypes depending on the relative capacity of the *VHL* mutants to regulate HIF, we sought to define the genotype-phenotype relationship regarding the capacity of *PHD2* mutants to differentially regulate HIF-α. Here we report a functional study comparing five *PHD2* variants associated with isolated congenital secondary erythrocytosis with the mutation identified in a patient with recurrent paraganglioma.

Design and Methods

Patients and mutation screening

Thirty-four patients who did not fulfill World Health Organization diagnostic criteria for polycythemia vera were investigated. The local ethics committee of Kremlin Bicetre Hospital approved the study and all patients provided written informed consent. Blood samples were collected from all patients and germline DNA was extracted and analyzed by direct sequencing.[20] DNA from the blood of healthy donors of Caucasian origin was used as a control.

Assay of hypoxia inducible factor transcriptional activity

In order to assay HIF transcriptional activity, dual luciferase assays were performed in Hek293T cells as described previously.[20] A pGL3promoter vector expressing luciferase under the control of hypoxia response elements[21] was used. Cells were exposed to hypoxic conditions (1% O_2) for 4 h before extraction.

Assay of hypoxia inducible factor stability

HeLa cells were transiently co-transfected with increasing amounts (50-200 ng) of an expression vector encoding the HIF-2α ODD domain (amino acids 404-569), fused to yeast Gal4 DNA-binding domain and Herpes simplex VP16-derived transactivation domain, together with the Gal4-response element driven firefly luciferase reporter, pGRE5xE1b (125 ng), and a Renilla luciferase control plasmid (4 ng).[22] Twenty-four hours post-transfection, cells were cultured under either normoxic (20% O_2) or hypoxic (1% O_2) conditions for an additional 16 h, and firefly luciferase activity was determined and normalized to Renilla luciferase activity.

Hydroxylation assay

PHD2 and HIF-ODD proteins (plasmid pcDNA3-HA-Gal4-HIF-1α-ODD was a generous gift from WG Kaelin Jr, Dana-Farber Cancer Institute, Boston, USA)[23] were produced in wheat germ extract *in vitro* using the TnT transcription-translation kit (Promega). The hydroxylation reaction was carried out by mixing PHD2 and HIF-1α-ODD (amino acids 536-652) proteins in a reaction buffer containing co-factors (Fe^{2+}, ascorbic acid and 2-oxoglutarate) as described previously.[24] The samples were incubated at 30°C, collected at different time points, and immunoblotted with anti-HA (Tebu) and anti-HIF-1α (Pro 564)-OH (Cell-Signaling Technology) antibodies.

Results

We sequenced the *PHD2* gene on germline DNA from a

series of Caucasian patients with unexplained polycythemia associated with normal or elevated serum erythropoietin levels (Figure 1A). Four novel heterozygous sequence variants were identified in the *PHD2* gene [c.G471C, p.Gln157His (Q157H); c.C599A, p.Pro200Gln (P200Q); c.G760C, p.Asp254His (D254H); c.C1192T, p.Arg398X (R398X)] as well as the already described c.G1112A, p.Arg371His (R371H) mutation[16] (Figures 1A and B). Genetic testing was performed on available parents and relatives of the *PHD2* mutation carriers, but no mutation was found except in the mother of the *PHD2*-R398X carrier who presented characteristics of polycythemia (Figure 1A). She harbors the *PHD2* mutation but with a mosaic status as demonstrated by the reduced height of the peak on the sequence chromatogram (Figure 1B). The low proportion of the mutated allele was confirmed by a quantitative allele-specific oligonucleotide method (*data not shown*).

Concerning the other families, only a few relatives were available for further genetic and clinical investigations but there was no history of familial polycythemia. Briefly, the brother, the sister and the son of patient #2295 and the father of patient #2403 agreed to genetic testing and were not carriers of *PHD2* mutation. Other parents had died previously, of known causes in two cases: esophageal cancer for the mother of patient #2403 and colon cancer for the mother of patient #2412. Finally, patient #0424 was an adopted child, without children.

The frequencies of the different variants were evaluated in a control population (Figure 1C). Only the Q157H variant was found and was, therefore, classified as a polymorphism. Analysis of amino acid conservation supports this conclusion, as the Q157 amino acid is not conserved either between species or within the PHD protein family (PHD1 and 3) (*Online Supplementary Table S1*). In contrast, amino acids P200 and R371 are highly conserved and the D254 amino acid is fully conserved, similar to H374 described in our previous study.[20] As far as concerns the location of the amino acids in the protein, D254, like H374, is part of the PHD2 catalytic site.[25] The P200 amino acid is located within the nuclear localization signal (NLS) implicated in the shuttling of PHD2 between the cytoplasm and the nucleus which plays a crucial role in HIF regulation.[26] We tested the shuttling of the P200Q mutation by immunofluorescence but we did not observe any impact of the mutation on the capacity of PHD2 to shuttle (*data not shown*).

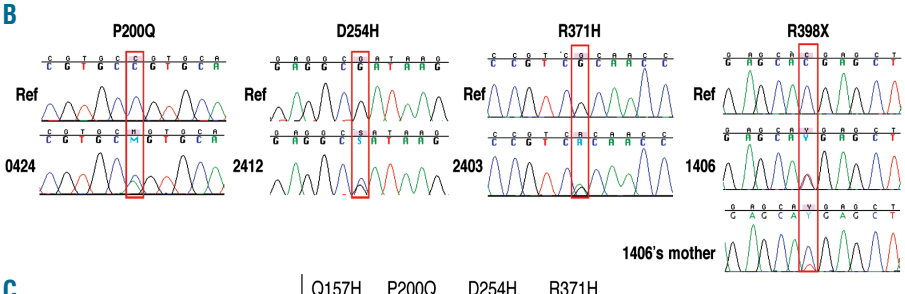
The functional consequences of these mutations on PHD2 activity were first evaluated using a hypoxia reporter assay based on the transcriptional activity of endogenous HIF (accumulated during 4 h of hypoxia). A luciferase reporter gene driven by a hypoxia response element derived from the erythropoietin 3' enhancer was used. Adding wild-type PHD2 led to a dose-dependent suppression of HIF-α-mediated luciferase expression (Figure 2A). As expected, the PHD2 variant with the Q157H polymorphism reduced luciferase activity to the same extent as wild-type PHD2 (Figure 2A). Within the different missense mutations, we observed two types of mutants: one type (including PHD2-P200Q and R371H) did not affect the capacity of PHD2 to regulate HIF-α transcriptional activity, whereas a second type (including PHD2-D254H and H374R) completely abrogated PHD2 activity (Figure 2A).

We next studied the *PHD2* mutants using a one-hybrid reporter assay based on the capacity of PHD2 to induce HIF-α protein instability. Cells were co-transfected with expression vectors containing the different PHD2 mutants (50 to 200 ng), the oxygen-dependent degradation domain of HIF-2α (HIF-2α-ODD) fused to yeast Gal4 DNA-binding domain and Herpes simplex VP16-derived transactivation domain, together with a reporter vector expressing luciferase under the control of a Gal4-response element. In this assay, luciferase expression reflects the stability of HIF-2α-ODD. A trend similar to that in the previous test was obtained: there was no detectable effect of the P200Q and R371H substitutions on PHD2 activity (which was compa-

A

Patient number	Mutation (nucleotide)	Mutation(protein)	Age	Age at the diagnosis of polycythemia	Sex	Ht (%) (Nm=40-52) (Nf=37-47)	Hb (g/dL) (Nm=13-18) (Nf=12-15)	RBC (10^6/mm^3) (Nm=4.2-5.7) (Nf=4.2-5.2)	EPO (mU/mL) (N=5-25)
8416	c.G471C	p.Gln157His (**Q157H**)	44	43	m	57.2	20.2	NA	24
0424	c.C599A	p.Pro200Gln (**P200Q**)	34	22	m	56	17.9	5.9	90
2412	c.G760C	p.Asp254His (**D254H**)	48	25	m	57.2	19.2	6.3	1.5-2 x N
2403	c.G1112A	p.Arg371His (**R371H**)	25	17	m	56.7	19.1	6.4	N
1406	c.C1192T	p.Arg398X (**R398X**)	41	26	m	53.8	19.3	5.9	6.5
1406's mother	c.C1192T	p.Arg398X (**R398X**)	67	64	f	49.5	16.1	5.2	NA
2295*	c.A1121G	p.His374Arg (**H374R**)	52	30	m	61.6	20.2	6.2	18

B



C

	Q157H	P200Q	D254H	R371H
Number of sequenced controls	173	173	188	133
Number of identified substitutions	5	0	0	0
Frequency	3%	0%	0%	0%

Figure 1. Identification of *PHD2* mutations in patients with polycythemia. (A) Table of patients diagnosed with erythrocytosis and a *PHD2* variation. Ht: hematocrit; Hb: hemoglobin; RC: number of red cells; EPO: erythropoietin; m: male; f: female; N: normal; NA: not available; *: previously described.[20] (B) Sequence chromatogram of *PHD2* in the area of the mutated nucleotide. Wild-type DNA was used as reference (Ref) (top) and compared to germline DNA of the patient (bottom). Only one relative of a patient was available for genetic testing (the mother of the *PHD2*-R398X carrier). She harbors the *PHD2* mutation but with a mosaic pattern as demonstrated by the reduced height of the peak on the sequence chromatogram. (C) Frequency of the *PHD2* variations in a control population.

rable to that of the wild-type PHD2) and there was total abolition of the D254H mutant activity (comparable to that of the truncated PHD2 variant R398X) (Figure 2B). Identical results were obtained using a HIF-1α-ODD stability reporter (*data not shown*). The experiment was repeated with the P200Q-PHD2 mutant in oxygen-regulated erythropoietin-expressing cells (including the human hepatoma cell line Hep3B and renal erythropoietin-producing cells, a new cell model isolated from the tumor-free tissue of a patient with renal carcinoma) but no significant difference was observed (*data not shown*).[27]

We next performed a sensitive *in vitro* assay in order to test the ability of the PHD2 mutants to hydroxylate HIF-1α in a time-dependent manner. In this assay, *in vitro*-translated PHD2 proteins were mixed with HIF-1α-ODD in the presence of co-factors necessary for the hydroxylation reaction. The capacity of the different PHD2 mutants to hydroxylate HIF-1α was measured by immunoblotting using an antibody specific for the hydroxylated HIF-1α-ODD (HIF-OH, Figure 3A). The H374R and D254H substitutions totally impaired HIF-1α hydroxylation (Figure 3A). By contrast the R371H mutant behaved like the wild-type PHD2 and the P200Q mutant, although capable of hydroxylating HIF-1α (Figure 3A), showed a reproducible and consistent delay (Figure 3B).

Discussion

Taken together, these results show that *PHD2* mutations can be divided into several different classes in terms of their effects on HIF regulation. Genotype/phenotype correlations cannot be established for *PHD2* mutations because they are rare events that have been reported in only ten families to date (including those in the present study), in contrast to the 945 families described with a *VHL* mutation.[10] In addition, the parents of the *PHD2* mutation carriers reported in the literature were either dead or not available for further genetic and clinical investigations (including one parent who died of esophageal cancer[18]). In our study, only one parent was genetically tested and diagnosed as a mosaic carrier which prevents any conclusion regarding the developed phenotype. Nonetheless, regarding the close functional relation between VHL and PHD2 in the regulation of HIF and the implication of the HIF pathway in the genesis of pheochromocytoma,[28-31] we can hypothesize similarities between the various types of mutants and raise the question of a possible risk of development of paraganglioma/pheochromocytoma in *PHD2* mutation carriers. Subjects with one category of mutantion (P200Q and R371H) display features similar to those with the VHL-R200W mutantion responsible for Chuvash polycythemia without any increased risk of neoplasia.[11] Like the VHL-R200W mutant, which is located outside functional domains, the PHD2-P200Q and R371H mutations are not located in the catalytic domain of the enzyme and have a moderate impact on HIFα regulation. In addition, the VHL-R200W mutant only induces delayed ubiquitination of HIFα which may be comparable to the delayed hydroxylation of HIFα observed with the PHD2-P200Q mutant. Interestingly, Pro200 is only one residue N-terminal to Cys201 which has been shown to chelate zinc and cadmium ions, providing evidence for the existence of a second metal binding site on PHD2.[32,33] This Cys201 affects PHD2 hydroxylation activity and appears to be implicated in redox signaling *in vitro*.[34] The very close location to the functionally important Cys201 residue could be the cause of the delayed hydroxylation of HIF-1α by the PHD2-

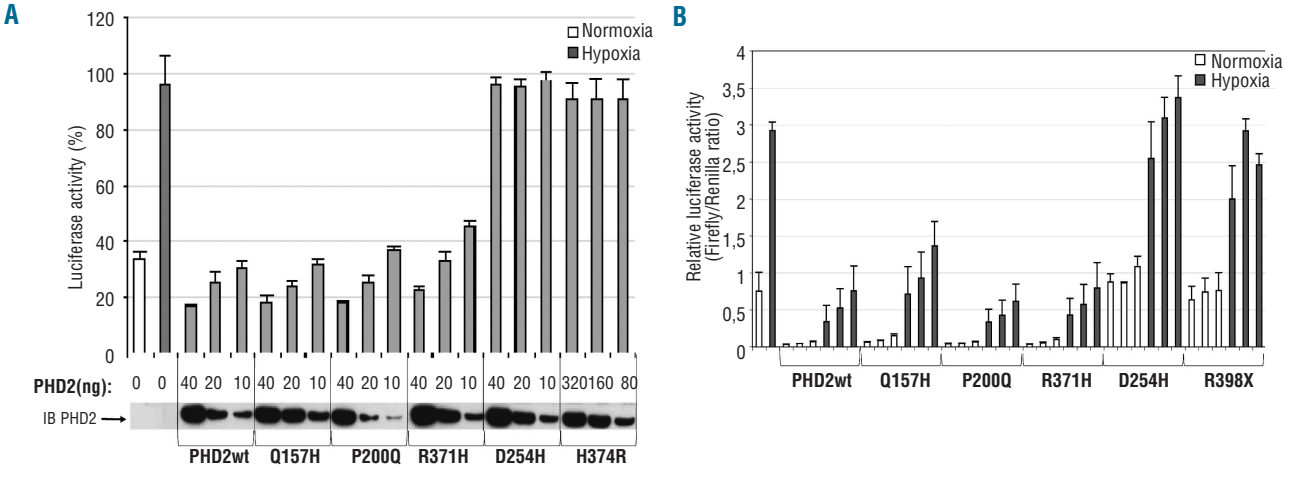


Figure 2. Functional study of *PHD2* mutants using luciferase reporter assays. (A) PHD2-dependent regulation of endogenous HIF in an assay based on a hypoxia response element reporter gene. Cells were co-transfected in a 12-well format with various amounts of pcDNA3-HA-PHD2 expression vectors (to enable the expression of the same amount of PHD2 proteins) in addition to pGL3 reporter vectors encoding firefly luciferase under the control of a sensitive hypoxia response element and *Renilla* luciferase as a control of transfection efficiency. Cells were placed in hypoxic conditions (1% O_2) for 4 h in order to accumulate endogenous HIFα before being collected. Results are given in percentage of firefly luciferase activity normalized to *Renilla* luciferase activity. The amount of HA-PHD2 transfected (PHD2) was quantified by immunoblotting using an anti-HA antibody. (B) The effect of PHD2 effect on HIF-2α protein stability in a one-hybrid reporter assay. Cells were cotransfected in a 6-well format with various amounts of pcDNA3-HA-PHD2 expression vectors (200, 100 and 50 ng), a Gal4-VP16-HIF-2αODD (amino acids 404-569) construct as well as a Gal4 response element-driven firefly luciferase reporter and a *Renilla* luciferase control plasmid. Twenty-four hours post-transfection cells were incubated for 16 h in normoxic or hypoxic conditions. Results are mean values of three independent experiments performed in triplicate.

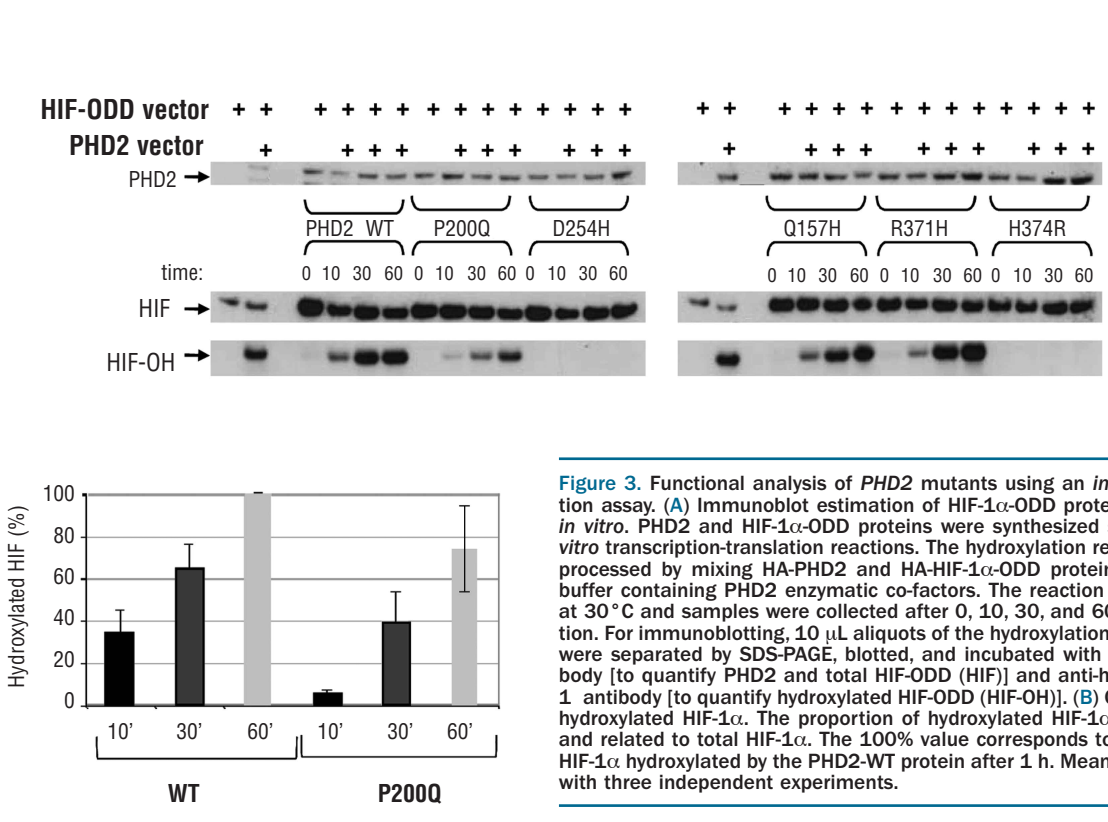


Figure 3. Functional analysis of *PHD2* mutants using an *in vitro* hydroxylation assay. (A) Immunoblot estimation of HIF-1α-ODD protein hydroxylation *in vitro*. PHD2 and HIF-1α-ODD proteins were synthesized separately by *in vitro* transcription-translation reactions. The hydroxylation reaction was then processed by mixing HA-PHD2 and HA-HIF-1α-ODD proteins in a reaction buffer containing PHD2 enzymatic co-factors. The reaction was carried out at 30°C and samples were collected after 0, 10, 30, and 60 min of incubation. For immunoblotting, 10 μL aliquots of the hydroxylation reaction assays were separated by SDS-PAGE, blotted, and incubated with an anti-HA antibody [to quantify PHD2 and total HIF-ODD (HIF)] and anti-hydroxylated HIF-1 antibody [to quantify hydroxylated HIF-ODD (HIF-OH)]. (B) Quantification of hydroxylated HIF-1α. The proportion of hydroxylated HIF-1α was measured and related to total HIF-1α. The 100% value corresponds to the quantity of HIF-1α hydroxylated by the PHD2-WT protein after 1 h. Means were obtained with three independent experiments.

P200Q mutant. Intriguingly, concerning the R371H mutant, the previously reported loss-of-function effect of this mutant[16] could not be confirmed by any of the three tests of our study. The R371H mutation segregates with erythrocytosis in two different families (described herein and by Percy *et al.*[16]) and is unequivocally involved in this pathology. We currently cannot explain why, in our hands, the R371H mutation failed to abolish PHD2 catalytic activity. The PHD2 expression vectors were re-sequenced and their functions confirmed by immunoblotting. Parallel experiments with other PHD2 mutants confirmed the validity of our assays. For this category of mutants which have a moderate impact on HIFα regulation, we cannot rule out potential indirect regulation on the oxygen sensing pathway via PHD2-interacting proteins. Indeed, during the past decade a large number of PHD2-interacting proteins have been discovered, including both upstream regulators and downstream targets of PHD2, substantially increasing the complexity of the PHD/HIF oxygen-sensing regulation pathway.[7] Moreover, PHD2 has been reported to have hydroxylation-independent gene regulatory functions.[35-37]

Another category, including the PHD2-R398X mutation and three other *PHD2* truncated mutations described previously,[12] can be compared to the *VHL* truncation mutations (VHL disease type 1) which are not associated with the development of pheochromocytomas. Subject with these first two categories could be considered at low risk of developing paraganglioma/pheochromocytoma.

A last category could be compared to the *VHL* missense mutations involved in VHL disease type 2, associated with a high risk of paraganglioma/pheochromocytoma. This category includes the previously described PHD2-H374R mutation identified in a 43-year-old patient with paraganglioma. A loss of the *PHD2* wild-type allele was demonstrated in the patient with this tumor, arguing for a tumor suppressor role of *PHD2*.[20] No *PHD2* mutations have been identified in other series of patients affected by pheochromocytomas (73 patients with hereditary paraganglioma and pheochomocytoma syndrome, Gimenez-Roqueplo, Hôpital Européen Georges Pompidou, Paris, *unpublished data*) and/or renal carcinoma,[38] but the risk that germline *PHD2* mutation carriers have of developing tumors should not be underestimated. Indeed, like His374, Asp254 is highly conserved, and located in the catalytic site of PHD2.[25] Moreover, the D254H mutation results in a severe loss of function. Therefore, although no cases of paraganglioma or pheochromocytoma have yet been detected in the D254H-*PHD2* mutation carrier, this mutation may be considered as a potential candidate for tumor predisposition.

In conclusion, using three different approaches we demonstrated that distinct *PHD2* mutations have differential effects on HIF regulation. We suggest that, by analogy to *VHL* mutations, carriers of particular *PHD2* mutations may be prone to tumor development. These patients would then require screening for tumor prevention and early detection.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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