Blood Cells, Molecules and Diseases 52 (2014) 35–45

Contents lists available at ScienceDirect

Blood Cells, Molecules and Diseases

journal homepage: www.elsevier.com/locate/bcmd

Iron deficiency modifies gene expression variation induced by augmented hypoxia sensing

Xu Zhang [a], Wei Zhang [b,c], Shwu-Fan Ma [d], Galina Miasniakova [e], Adelina Sergueeva [f], Tatiana Ammosova [g], Min Xu [g], Sergei Nekhai [g], Mehdi Nourai [g], Michael S. Wade [h,i], Josef T. Prchal [j,k,l], Joe G.N. Garcia [h,i,1], Roberto F. Machado [h,i,1], Victor R. Gordeuk [a,*,1]

[a] Comprehensive Sickle Cell Center, Section of Hematology/Oncology, Department of Medicine, University of Illinois at Chicago, Chicago, IL, USA
[b] Department of Pediatrics, University of Illinois at Chicago, Chicago, IL, USA
[c] Institute of Human Genetics, University of Illinois at Chicago, Chicago, IL, USA
[d] Section of Pulmonary/Critical Care, Department of Medicine, University of Chicago, Chicago, IL, USA
[e] Chuvash Republic Clinical Hospital 2, Cheboksary, Russian Federation
[f] Cheboksary Children's Hospital, Cheboksary, Russian Federation
[g] Center for Sickle Cell Disease, Howard University, Washington, DC, USA
[h] Section of Pulmonary, Critical Care & Sleep Medicine, Department of Medicine, University of Illinois at Chicago, Chicago, IL, USA
[i] Institute for Personalized Respiratory Medicine, University of Illinois at Chicago, Chicago, IL, USA
[j] Department of Medicine, University of Utah and VAH, Salt Lake City, UT, USA
[k] Department of Pathology, University of Utah and VAH, USA
[l] Department of Genetics, University of Utah and VAH, USA

ARTICLE INFO

Article history:
Submitted 19 July 2013
Available online 28 August 2013

(Communicated by J.T. Prchal, M.D., 19 July 2013)

Keywords:
Hypoxia-sensing
Hypoxia inducible factor
Iron deficiency
VHL
Chuvash polycythemia
Gene expression

ABSTRACT

In congenital Chuvash polycythemia (CP), VHL^{R200W} homozygosity leads to elevated hypoxia inducible factor (HIF) levels at normoxia. CP is often treated by phlebotomy resulting in iron deficiency, permitting us to examine the separate and synergistic effects of iron deficiency and HIF signaling on gene expression. We compared peripheral blood mononuclear cell gene expression profiles of eight VHL^{R200W} homozygotes with 17 wildtype individuals with normal iron status and found 812 up-regulated and 2120 down-regulated genes at false discovery rate of 0.05. Among differential genes we identified three major gene regulation modules involving induction of innate immune responses, alteration of carbohydrate and lipid metabolism, and down-regulation of cell proliferation, stress-induced apoptosis and T-cell activation. These observations suggest molecular mechanisms for previous observations in CP of lower blood sugar without increased insulin and low oncogenic potential. Studies including 16 additional VHL^{R200W} homozygotes with low ferritin indicated that iron deficiency enhanced the induction effect of VHL^{R200W} for 50 genes including hemoglobin synthesis loci but suppressed the effect for 107 genes enriched for HIF-2 targets. This pattern is consistent with potentiation of HIF-1α protein stability by iron deficiency but a trend for down-regulation of HIF-2α translation by iron deficiency overriding an increase in HIF-2α protein stability.

Introduction

Hypoxia [1] and iron [2] modulate many metabolic processes. Chronic and acute hypoxia cause morbidity and mortality associated with pulmonary and brain edema [3], pulmonary hypertension [4] and aberrant metabolism [5–7]. Hypoxia has multiple effects on the expression of a vast array of genes, but this has been almost entirely investigated in vitro [8] or in experimental animals [9]. Iron deficiency is a common nutritional disorder, and it enhances pathways that are associated with hypoxia as iron is required for optimal activity of prolyl hydroxylases (PHDs) that are principal negative regulators of hypoxia inducible factors (HIFs) [10,11]; iron deficiency also has hypoxia-unrelated metabolism-regulating roles [12–16]. Investigating the relationship between gene expression and the nutritional environment is important for understanding the complications of genetic disorders and for the development of optimal personalized approaches to medical care [17].

To better understand the pathological processes associated with hypoxia and to develop targeted intervention for disease states associated with hypoxia, we elected to define the hypoxia- and iron-related regulation of genes in humans in vivo by taking advantage of a congenital disorder of up-regulation of the hypoxic response at normoxia. Chuvash polycythemia (CP) is an autosomal recessive form of polycythemia/

* Corresponding author at: Suite 172, Clinical Sciences Building, 820 South Wood St., Chicago, IL 60612-7236, USA. Fax: +1 312 996 5984.
E-mail address: vgordeuk@uic.edu (V.R. Gordeuk).
[1] Contributed equally to this work as senior authors.

1079-9796/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bcmd.2013.07.016

erythrocytosis that is endemic to the mid-Volga River region in the Russian Federation [18] and is characterized by increased levels of HIFs during normoxia [19]. Furthermore, CP is often accompanied by iron deficiency due to therapeutic phlebotomies, allowing us to weigh the effect of this common nutritional and environmental variable on hypoxic gene expression.

HIF-1 and HIF-2 are transcription factors that serve as master regulators of the hypoxic response. With normal oxygen tension, von Hippel–Lindau (VHL) protein binds to HIF-α subunits and labels them for degradation by proteasomes. Proline hydroxylation of HIF-α by PHD enzymes is required for the interaction of HIF-α with VHL protein [20,21]. Both oxygen and iron are needed for the activity of PHDs. Low oxygen inhibits VHL binding to HIF-α and activates HIF-dependent transcriptional responses, including increased erythropoiesis, angiogenesis, and a metabolic shift from oxidative phosphorylation to glycolysis for ATP generation. HIF-1α and HIF-2α may have complimentary but in some contexts also opposite effects on distinct gene targets depending on tissue and cell types [22,23]. Iron deficiency also has the potential to exacerbate the hypoxic response due to its effect of impairing the activity of PHDs. Consistent with this possibility, iron chelating drugs post-translationally increase the α subunits of HIFs in cultured cells [10,11], likely via PHD inhibition and apparently overriding a possible confounding effect of lack of iron in decreasing HIF-2α translation by promoting iron response proteins [24].

CP is characterized by a homozygous 598C>T (Arg200Trp or R200W) germline missense mutation in the *VHL* gene [19,22], which leads to impaired activity of VHL protein to initiate ubiquitination and ultimately degradation of HIF-1α and HIF-2α. Patients with CP have increased levels of HIF-1 and HIF-2 during normoxia; this leads to altered expression of a large array of HIF-target genes, still not fully defined, and clinical manifestations that have so far been shown to include elevated hematocrit, lower systolic blood pressure (SBP) and enhanced risk of hemorrhage, thrombosis, pulmonary hypertension and other complications [25,26]. In CP subjects iron deficiency would be predicted to further increase HIF-1α and HIF-2α and augment the increased HIF activity that occurs due to the genetic loss of VHL function [19,20]; however, the opposite effect on HIF-2α mediated by its 5′ iron responsive element [24] is also possible. The study of CP subjects might further clarify the role of iron deficiency in manifestations of the hypoxic response.

In this study we compared gene expression variation in peripheral blood mononuclear cells (PBMCs) between 24 VHL^{R200W} homozygotes and 21 Chuvash *VHL* wildtype controls. Those VHL^{R200W} homozygotes on a therapeutic phlebotomy are almost universally iron deficient. We therefore selected 17 healthy Chuvash *VHL* wildtype (WT) and 8 VHL^{R200W} homozygotes matched for serum ferritin concentration, and identified 2932 differentially expressed genes and related biological pathways attributable to the VHL^{R200W} mutation. We found that iron deficiency affected VHL^{R200W} induced gene expression possibly by distinct mechanisms.

Materials and methods

Study subjects

The Howard University IRB committee approved the protocol and each participant gave written informed consent. Twenty-one healthy Chuvash *VHL* wildtype (WT) control individuals and 24 Chuvash VHL^{R200W} homozygotes were recruited. The complete blood count was determined by an automated analyzer (Sysmex XT 2000i, Sysmex Corporation, Kobe, Hyogo, Japan); serum ferritin, serum erythropoietin (EPO), and plasma vascular endothelial growth factor (VEGF) concentrations by enzyme linked immunosorbent assay (ELISA) (Ramco Laboratories Inc., Stafford, TX and R&D Systems, Minneapolis, MN).

RNA isolation and expression profiling

PBMCs were isolated from EDTA whole blood by Ficoll-Hypaque (GE, Pittsburgh, PA) density gradient centrifugation, washed with PBS, re-suspended in *Ambion RNAlater* (Invitrogen, Carlsbad, CA) solution, frozen at −80 °C and shipped in liquid nitrogen from Cheboksary (Chuvashia, Russia) to Howard University. RNA was isolated using TRIzol reagent (Invitrogen, CA) and the quality was assessed using nanodrop (Thermo Scientific, Waltham, MA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Total RNA was submitted to the University of Chicago Functional Genomics Center for whole transcript sense target labeling assay (Affymetrix, Inc., Santa Clara, CA), hybridization to the *Affymetrix GeneChip*™ Human Exon 1.0 ST Array, and washing and scanning according to the manufacturer's procedure (Affymetrix, Inc., Santa Clara, CA).

Microarray data preprocessing

All 25mer probe sequences were aligned to human genome assembly GRCh37 allowing ≤2 mismatches [27]. Probes with perfect unique match to the genome were selected. We further removed probes that interrogated multiple gene transcripts and that contained SNPs with ≥1% minor allele frequency in the dbSNP dataset (v135) [28]. Probe level intensities were $\log_2$ transformed, background corrected [29] and quantile normalized [30]. Probe intensity was subtracted by the corresponding probe mean across samples. Gene-level expression intensities were summarized as mean probe intensity within each

Table 1
Comparison of clinical variables in VHL^{R200W} homozygotes and *VHL* wildtype controls. Results in median (interquartile range) are presented unless otherwise indicated. Wilcoxon's rank sum test was applied in all comparisons except in gender, in which Fisher's exact test was used. Subjects with ferritin concentration ≥ 21 μg/l are presented in B, where ferritin concentration ranges from 24 to 156 μg/l in control individuals and from 23 to 131 μg/l in VHL^{R200W} homozygotes ($P = 0.4$).

	WT		VHL^{R200W}		*P*
	N	Median (IQR)	N	Median (IQR)	
A. All subjects					
Age (years)	21	36 (31, 54)	24	35.5 (26, 52)	0.4
Female gender, n (%)	21	12 (57%)	24	15 (63%)	0.8
Systolic blood pressure (mm Hg)	21	120 (114, 125)	23	112 (105, 122)	0.1
Diastolic blood pressure (mm Hg)	21	81 (76, 85)	23	78 (72, 85.5)	0.6
Pulse pressure (mm Hg)	21	40 (35, 43)	23	39 (30, 43)	0.2
Red blood cells (1000000/μl)	21	4.5 (4.2, 4.9)	24	6.9 (6.4, 7.8)	<0.001
Hemoglobin (g/l)	21	125 (121, 139)	24	183 (155, 191)	<0.001
Hematocrit (%)	21	37.4 (36.5, 41.5)	24	56.2 (50.2, 58.6)	<0.001
Platelet (1000/μl)	21	248 (213, 301)	23	220 (189, 286)	0.3
Ferritin (μg/l)	19	58 (26, 76)	24	14 (7, 25)	0.001
Erythropoietin (IU/l)	19	7.4 (5.4, 10.5)	23	48.4 (20, 75.1)	<0.001
Vascular endothelial growth factor (pg/ml)	16	0.2 (0.2, 0.3)	23	0.2 (0.2, 5.7)	0.039
B. Subset matched for ferritin					
Age (years)	17	40 (31, 56)	8	48 (34, 53)	0.9
Female gender, n (%)	17	9 (53%)	8	5 (63%)	1
Systolic blood pressure (mm Hg)	17	120 (118, 125)	8	109 (104, 126)	0.2
Diastolic blood pressure (mm Hg)	17	82 (78, 85)	8	81 (77, 91)	0.9
Pulse pressure (mm Hg)	17	40 (35, 47)	8	31 (25, 40)	0.047
Red blood cells (1000000/μl)	17	4.5 (4.2, 5)	8	6.9 (6.2, 7.8)	<0.001
Hemoglobin (g/l)	17	128 (124, 141)	8	187 (160, 191)	<0.001
Hematocrit (%)	17	39.5 (36.5, 42.8)	8	54.5 (50.2, 58.6)	<0.001
Platelet (1000/μl)	17	251 (213, 301)	8	248 (179, 292)	0.5
Ferritin (μg/l)	17	62 (29, 78)	8	46 (28, 64)	0.4
Erythropoietin (IU/l)	17	7.2 (5.4, 10.1)	8	50 (18.5, 79.3)	<0.001
Vascular endothelial growth factor (pg/ml)	12	0.2 (0.2, 0.3)	8	4.6 (0.2, 14.4)	0.025