Increased size of solid organs in patients with Chuvash polycythemia and in mice with altered expression of HIF-1α and HIF-2α

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Abstract

Chuvash polycythemia, the first hereditary disease associated with dysregulated oxygen-sensing to be recognized, is characterized by a homozygous germ-line loss-of-function mutation of the *VHL* gene (VHL^{R200W}) resulting in elevated hypoxia inducible factor (HIF)-1α and HIF-2α levels, increased red cell mass and propensity to thrombosis. Organ volume is determined by the size and number of cells, and the underlying molecular control mechanisms are not fully elucidated. Work from several groups has demonstrated that the proliferation of cells is regulated in opposite directions by HIF-1α and HIF-2α. HIF-1α inhibits cell proliferation by displacing MYC from the promoter of the gene encoding the cyclin-dependent kinase inhibitor, $p21^{Cip1}$, thereby inducing its expression. In contrast, HIF-2α promotes MYC activity and cell proliferation. Here we report that the volumes of liver, spleen, and kidneys relative to body mass were larger in 30 individuals with Chuvash polycythemia than in 30 matched Chuvash controls. In $Hif1a^{+/-}$ mice, which are heterozygous for a null (knockout) allele at the locus encoding HIF-1α, hepatic HIF-2α mRNA was increased (2-fold) and the mass of the liver was increased, compared with wild-type littermates, without significant difference in cell volume. Hepatic $p21^{Cip1}$ mRNA levels were 9.5-fold lower in $Hif1a^{+/-}$ mice compared with wild-type littermates. These data suggest that, in addition to increased red cell mass, the sizes of liver, spleen, and kidneys are increased in Chuvash polycythemia. At least in the liver, this phenotype may result from increased HIF-2α and decreased $p21^{Cip1}$ levels leading to increased hepatocyte proliferation.

Keywords

Introduction

Chuvash polycythemia (familial erythrocytosis, type 2, OMIM #263400), the first hereditary disease associated with augmented hypoxia-sensing to be recognized, is an autosomal recessive disorder with increased red cell mass [1]. While congenital polycythemia is sporadic worldwide, hundreds of patients with Chuvash polycythemia are found in the Chuvash population of central Russia [1,2] and the condition is also endemic on the Italian island of Ischia [3]. Chuvash polycythemia is due to homozygosity for the *598C>T* missense mutation in the von Hippel–Lindau gene (*VHL*) that results in the substitution of tryptophan for arginine at codon 200 (designated R200W) [4]. The VHL protein, which is the recognition component of an E3 ubiquitin-protein ligase complex, mediates proteasomal degradation of HIF-1α and HIF-2α under normoxic conditions [5]. HIF-1α and HIF-2α are subject to hydroxylation on specific proline residues by prolyl hydroxylase domain protein 2 (PHD2), an enzyme that requires oxygen as a substrate, and proline hydroxylation is required for the interaction of HIF-1α and HIF-2α with VHL [6,7]. Thus, HIF-1α and HIF-2α levels rise in response to hypoxia and this leads to increased expression of erythropoietin [8] and other hypoxia-inducible genes [7]. Homozygosity for the VHL^{R200W} allele leads to impaired interaction of VHL with both HIF-1α and HIF-2α proteins, increased levels of these HIFs in normoxia, and altered expression of multiple HIF-regulated genes

including increased serum levels of erythropoietin [2,4,9,10]. In addition to polycythemia, affected individuals may manifest altered cardiovascular and ventilatory responses and pulmonary hypertension [2,10,11].

Organ size is determined by cell growth, proliferation, and survival [12]. The sizes of the spleen [13] and liver [14] appear to be regulated by humoral factors active in embryonic as well as in adult life. The number of hematopoietic stem cells and hepatocytes is determined by both intrinsic and humoral factors [15,16], and circulating cytokines can induce rapid regeneration of the bone marrow and liver [17]. Humoral factors that regulate organ size include growth hormone, insulin, insulin-like growth factor, thyroid hormone, sex hormones, and cytokines [17–19]. Intrinsic factors that lead to growth restraints on organs and that determine organ size during a set point in embryonic development are largely unknown [14]. Several of the humoral factors that regulate organ size also modulate the expression of HIF-1α and HIF-2α [20,21].

Hypoxia influences proliferation of mammalian cell cultures as well as in animal models [22]. Under hypoxic conditions, HIF-1α induces cell cycle arrest associated with decreased CDK2 activity and hypo-phosphorylation of the retinoblastoma protein. This arrest is mediated in part by HIF-1α-induced expression of *p21^{Cip1}*, an inhibitor of cyclin-dependent kinases. Under hypoxic conditions, HIF-1α displaces MYC on the *p21^{Cip1}* promoter and activates gene transcription [23]. In contrast, HIF-2α promotes cell proliferation in various cell lines under hypoxic conditions by stimulating MYC binding to its target gene promoters [24]. Mice in which the *Vhl* gene is conditionally disrupted in hepatocytes have increased levels of HIF-2α, increased target gene expression, hepatomegaly, and reduced body weight [25]. Transgenic mice expressing a mutant HIF-2α protein that is resistant to prolyl hydroxylation and VHL-mediated degradation have hepatomegaly but not decreased body weight [26]. The goal of the present study was to determine if, in addition to increased red blood cell mass, the constitutional up-regulation of HIF-1α and HIF-2α in Chuvash polycythemia is associated with increased size of solid organs.

Materials methods

Study subjects and determination of organ size by computerized tomography

Thirty patients with Chuvash polycythemia and 32 control residents of Chuvashia, Russia were studied at a time when they were ambulatory and in their usual state of health. The research in humans was approved by the IRB of Howard University and by Chuvash Republic Cardiac Center, Cheboksary, Chuvashia, Russia, and written informed consent was obtained from all participants. Thirty of the controls were matched by sex and age to the patients with Chuvash polycythemia. Computerized tomography was performed with 5-mm sections using helical mode. After obtaining preliminary non-enhanced scans, approximately 120 ml of non-ionic iodinated contrast agent was injected and arterial and venous phase images were obtained. The volumes of the spleen, liver and kidneys were measured using the disc summation method with DicomWorks v1.3.5 software. The area of each axial slice of the organ was measured, the resultant areas were then added, and then the sum of the areas was multiplied by the thickness of each image (5 mm).

PCR of the VHL^{R200W} mutation

Genomic DNA was isolated using a QIAGEN column (QIAGEN Inc, Valencia, CA) and PCR reactions were performed in 50 µl containing 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl$_2$, 100 µM dNTP, 300 nM primers, and 2.5 U/reaction *Taq* DNA polymerase (Life Technologies, Grand Island, NY). The following primers were used for amplification of *VHL* exon 3: *VHL*3F, 5′-CCTTGTACTGAGACCCTAG; *VHL*3R, 5′-

GCTGAGATGAAACAGTGTA. Ten microliters of PCR product were incubated with 5 U of *Fnu4H*I (New England Biolabs Inc, Beverly, MA) for 2 h to detect the *VHL^R200W* mutation. The *VHL598C> T* (*VHL^R200W*) mutation abolishes a restriction site for *Fnu4H*I, resulting in an uncut 296-base-pair band detected by 1.2% agarose gel electrophoresis [4]. We confirmed the validity of this methodology on 88 additional genomic DNA samples by also performing allelic specific PCR using an ABI custom designed probe/primer mix (Applied Biosystems, Foster City, CA). Genomic DNA, 50 ng, was used for allele specific PCR and the reaction was carried out at 50°C for 2 min, 95°C for 10 min, followed by 45 cycles at 92°C for 15 s and 60°C for 1 min. There was an absolute concordance of results in 51 *VHL^R200W* homozygotes, 4 heterozygotes and 33 *VHL* wildtype samples that were examined by both methods.

Hif1a$^{+/-}$ mice and wild-type littermates

Hif1a$^{+/-}$ mice were generated and maintained as previously described [27]. The animal experimentation conformed to protocols approved by the animal care committees of the University of Utah. *Hif1a*$^{+/-}$ mice and wild-type littermates older than 12 weeks of age were weighed, euthanized, and then dissected to remove the liver, spleen and kidneys. These organs were weighed in 1 ml of PBS and the weight was expressed as percentage of total body weight. One third of one lobe of each liver was fixed overnight, embedded in paraffin, and cut into 6-µm-thick sections and stained with hematoxylin and eosin followed by dewaxing and rehydration. Hepatocyte area was measured using ImageJ software (NIH).

Total RNA was extracted from livers of *Hif1a*$^{+/-}$ mice and wild-type littermates using Trizol (Molecular Research Center, Cincinnati, OH). Three aliquots of total RNA (300 ng) from each littermate were collected and pooled and analyzed at the University of Utah Microarray Core Facility using the Affymetrix Microarray Platform (Affymetrix, Santa Clara, CA). Results were analyzed by the University of Utah Bioinformatics Core. To quantify HIF-2α and p21^{cip1} mRNA levels, hepatic RNA (500 ng) was reverse transcribed into cDNA using SuperScript II with oligo dT primer according to the manufacturer's instructions (Invitrogen, Carlsbad, CA) and 2µl of cDNA was used for real-time PCR (ABI 7000, Applied Biosystems, Foster City, CA). For HIF-2α mRNA and 18S rRNA, we used SYBR Green dye with specific primers (Hif-2α: 5′-CTT GTA CCT GAA AGC CTT GG-3′ and 5′-GTC CCA TGA ACT TGC TGA TG-3′; 18S: 5′-TTG ACG GAA GGG CAC CAC CAG-3′ and 5′-GCA CCA CCA CCC ACG GAA TCG-3′) and the reaction was carried out at 50°C for 2 min, 95°C for 10 min, followed by 50 cycles of 92°C for 15 s and 58°C for 1 min. For *p21^Cip1* we used a Taqman probe (Assay ID, Mn01303209_m1) and followed the manufacturer's suggested protocol (ABI, Carlsbad, CA). ΔCt was expressed after normalization against 18S. Fold change was calculated by the ΔΔCt method [28].

Statistical analysis

Continuous variables were compared between Chuvash polycythemia patients and controls with the paired Student's *t* test. Organ volumes were also compared between patients and controls in multiple linear regression models. Mouse organ/body mass ratios were compared with the Kruskal–Wallis test.

Results

Organ size in Chuvash polycythemia and unaffected control subjects

Clinical features—*VHL^R200W* homozygosity was confirmed by PCR in 23 of the 30 subjects with the diagnosis of Chuvash polycythemia, whereas in seven polycythemic subjects DNA was not available. *VHL* wild-type status was confirmed by PCR in 27 of the control subjects, two control subjects were *VHL^R200W* heterozygotes, and DNA was not

available for three controls. In addition to higher hemoglobin concentration, the Chuvash polycythemia subjects had lower body weight, body mass index, and systolic blood pressure compared to controls (Table 1) as previously reported [2,10].

Organ volumes—Despite decreased body weight, the 30 subjects with Chuvash polycythemia had larger volumes of the liver, spleen, and kidneys compared to 30 age- and sex-matched controls, as determined by computerized tomography and the disc summation method (Table 1). Expressed as the ratio of organ volume to body mass, the mean volume of the liver in Chuvash polycythemia patients was greater than in controls by a factor of 1.2 (95% confidence interval of 1.1 to 1.3; P=0.0006), the mean volume of the spleen was greater by a factor of 2.3 (1.8 to 2.8; P=0.0001) and the mean volume of the kidneys was greater by a factor of 1.2 (1.1 to 1.4; P=0.005). The present study was conducted in adults, and therefore reflects the size of the body and selected organs after the development that occurs during the embryonic, fetal and childhood periods of life.

Because of recognized relationships of larger organ size with greater body mass, [29] larger organ size with exposure to androgens [30], and decreasing organ size with advancing age [31], organ volume was examined among patients with Chuvash polycythemia and controls with multiple linear regression analyses that included body mass, sex, and age (Table 2). As expected [29], increased body weight was independently associated with increased volumes of the liver, spleen, and kidneys (P≤0.003) and male sex with increased volumes of the liver and kidneys (P=0.001). Consistent with some [32] but not all studies [31], liver and kidney size did not decrease with more advanced age, and spleen size did not decrease with female sex or age. Chuvash polycythemia was associated with an increase in liver volume comparable to a 13 kg greater body mass (P<0.0005), an increase in spleen volume comparable to a 42 kg greater body mass (P<0.0001), and an increase in kidney volume comparable to a 13 kg greater body mass (P<0.05). In similar analyses that included body mass index rather than body weight as an explanatory variable, the adjusted liver volume (P<0.001), spleen volume (P<0.0005), and total kidney volume (P<0.05) continued to be larger in patients with Chuvash polycythemia compared to controls.

Analysis of HIF-1α-deficient mice

Organ size of Hif1a$^{+/−}$ mice and Hif1a$^{+/+}$ littermates—Since we demonstrated increased HIF-1α levels in Chuvash polycythemia patients homozygous for the VHL^{R200W} allele [4] and other investigators demonstrated increased HIF-2α levels in mice carrying this mutation [9], we hypothesized that increased HIF activity may contribute to larger organ volume in VHL^{R200W} homozygotes. To test this hypothesis, the organ to body mass ratio was determined in $Hif1a^{+/−}$ mice, which are heterozygous for a null allele at the locus encoding HIF-1α, [27] and their wild-type littermates. As shown in Table 3, $Hif1a^{+/−}$ mice had increased median organ-to-body mass ratio for the liver (factor of 1.5, P<0.005) relative to wild-type littermates. Hepatocyte area was not significantly different in $Hif1a^{+/−}$ mice and their wild-type littermates (Fig. 1), suggesting that an increase in cell number rather than cell volume was responsible for the increased mass of $Hif1a^{+/−}$ livers.

Gene expression—To explore potential molecular mechanisms underlying the increased liver-to-body mass ratio in $Hif1a^{+/−}$ mice, total RNA was extracted from livers of $Hif1α^{+/−}$ mice and wild-type littermates, and gene expression profiles were performed using Affymetrix microarrays. This procedure revealed that 2,167 genes were down-regulated more than two-fold in $Hif1a^{+/−}$ mice compared to wild-type mice genes and 1,628 genes were up-regulated more than two-fold. Among these differentially expressed genes were HIF-2α, which was consistently up-regulated, and $p21^{Cip1}$, which was consistently down-regulated. The changes of expression of $p21^{Cip1}$ and HIF-2α were confirmed using

quantitative real-time RT-PCR. As shown in Fig. 2, HIF-2α mRNA levels were increased 2-fold ($P<0.05$) and $p21^{Cip1}$ mRNA levels were decreased 9.5-fold ($P=0.05$) in the livers of $Hif1\alpha^{+/-}$ mice compared to wild-type littermates. These findings are consistent with the hypothesis that HIF-regulated pathways are important in determining the volume of body organs [20,21], specifically by a pathway in which increased HIF-2α expression leads to induction of MYC activity, inhibition of $p21^{Cip1}$ expression and a resultant increase in cell proliferation [23,24].

Discussion

Previous studies indicated that homozygosity for the VHL^{R200W} mutation is associated with elevated hemoglobin concentration, cerebral vascular events, peripheral thrombosis, benign vertebral hemangiomas, varicose veins, heart murmurs, lower systemic blood pressure, elevated pulmonary artery pressure, increased expression of HIF-regulated genes, elevated levels of homocysteine and glutathione, altered inflammatory cytokine profiles, and no increased risk of malignancies associated with the von Hippel–Lindau syndrome [2,10,11,33,34]. Our present work indicates that volumes of the liver, spleen, and kidneys, as adjusted for body weight, sex, and age, are significantly greater in patients with Chuvash polycythemia compared with control subjects, and that like polycythemia, this may be due to increased cell proliferation.

Past studies demonstrating that HIF-1α decreases cell proliferation by inducing $p21^{Cip1}$ expression [23] and that HIF-2α induces MYC activity and cell proliferation [24] were carried out in various tumor cells. Our present findings potentially relate these pathways to the larger size of the liver in $Hif1a^{+/-}$ compared with $Hif1a^{+/+}$ mice. Our previous work showed a higher level of HIF-1α in EBV-transformed lymphocytes of Chuvash polycythemia patients although HIF-2α levels were not tested [4]. Recently, mice bearing the Vhl^{R200W} gene were generated by homologous recombination and found to recapitulate many features of human Chuvash polycythemia, including elevated hematocrit and serum erythropoietin levels. Moreover, in the livers of Vhl^{R200W} homozygous mice, HIF-2 target gene transcripts were preferentially up regulated compared to HIF-1 target gene transcripts [9]. Taken together with published data, our present observations suggest that, at least in the liver of patients with Chuvash polycythemia, the HIF-1α:HIF-2α ratio may be altered to favor HIF-2α, and that increased HIF-2α levels may lead to suppression of $p21^{Cip1}$ expression, resulting in increased proliferation of hepatocytes and larger organ volume to body mass ratio.

There are a number of limitations to our study. Firstly, the diagnosis of Chuvash polycythemia was not confirmed by genotyping in seven of 30 patients. However, the clinical diagnosis of Chuvash polycythemia by physicians in Chuvashia is highly accurate in predicting VHL^{R200W} homozygous genotype. Of 150 patients with the diagnosis of Chuvash polycythemia who we have genotyped to date, 142 have been VHL^{R200W} homozygotes, two VHL^{R200W} heterozygotes and six VHL wildtype individuals (Gordeuk 2010, unpublished data). Secondly, the genotype of three of the 32 controls was not determined. In our studies to date, none of 449 persons in Chuvashia without the diagnosis of Chuvash polycythemia have proved to be VHL^{R200W} homozygotes when genotyped (Gordeuk 2010, unpublished data). Thirdly, two of the 29 controls who were genotyped proved to be VHL^{R200W} heterozygotes. The study was designed to select both patients and controls on a clinical basis, before genotyping was performed. In our studies to date, heterozygotes have not had significantly increased hemoglobin concentrations compared to controls [2]. Fourthly, the study did not match cases and controls according to body mass index, and the controls had a slightly but significantly higher body mass index compared to cases. However, in multivariate analysis that were adjusted either for body weight or for body mass index, the

least square mean volumes of the liver, spleen and kidneys were significantly greater in patients with Chuvash polycythemia compared to controls. The lower body mass index associated with Chuvash polycythemia observed here is consistent with our previous work [2] and may reflect the altered expression of multiple HIF-regulated genes in the setting of homozygosity for the *VHL^R200W* allele.

Whether there are any selective advantages or disadvantages to larger size of solid organs in association with Chuvash polycythemia is unclear. Amechanismof enhanced cellular proliferation due to HIF-2α-induced suppression of *p21^Cip1* expression may contribute not only to larger organ size but also other manifestations of Chuvash polycythemia. Erythroid progenitors in Chuvash polycythemia display heightened sensitivity to erythropoietin [4], suggesting that other mechanisms in addition to increased erythropoietin levels contribute to the increased proliferation of erythroid progenitors. Pulmonary hypertension is a complication of Chuvash polycythemia [10,11], the pathophysiology of which includes increased proliferation and hypertrophy of pulmonary vascular smooth muscle cells [35].

Acknowledgments

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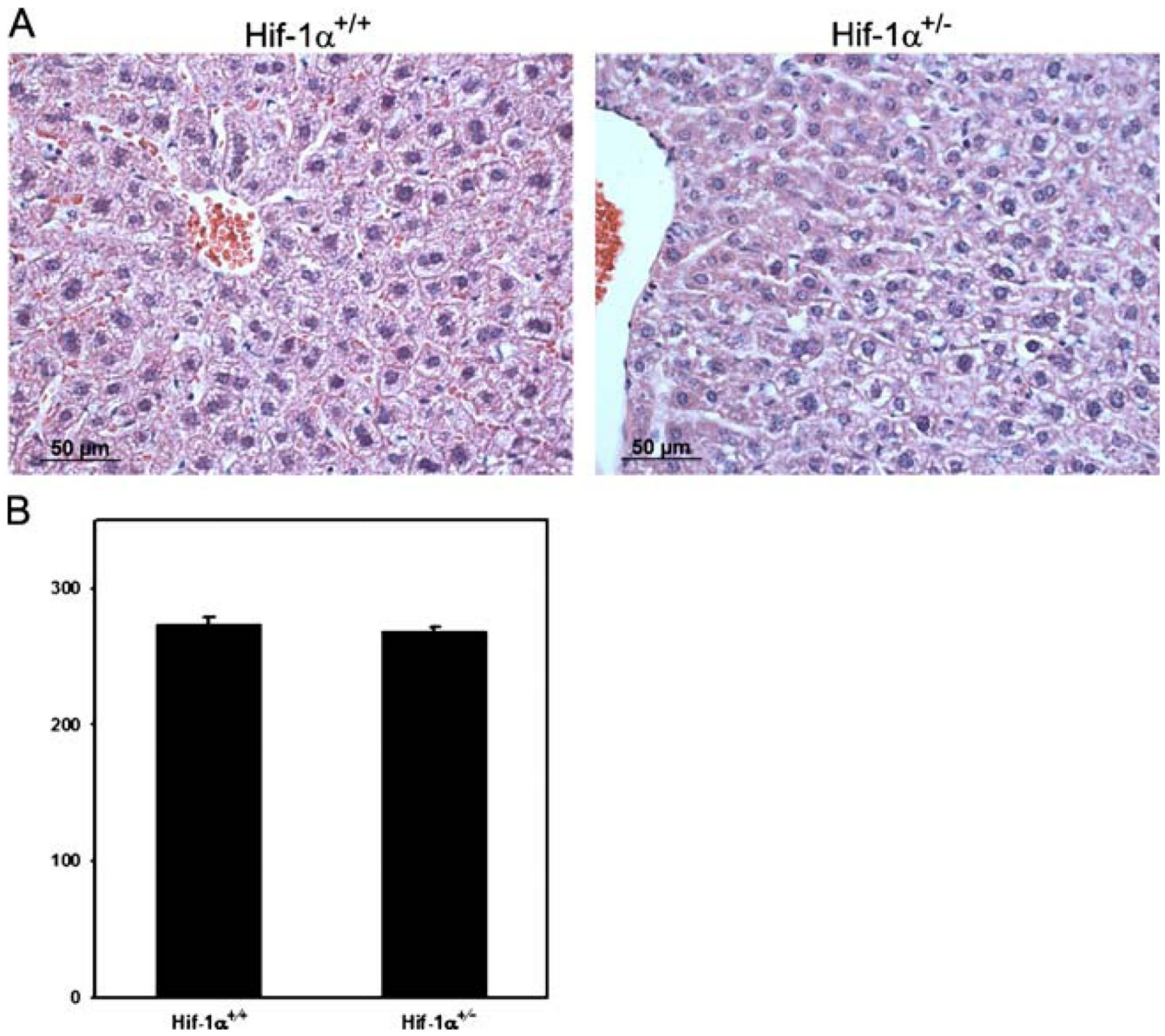
Fig. 1.
Hematoxylin- and eosin-stained sections of livers from $Hif1a^{+/+}$ and $Hif1a^{+/-}$ mice. **a** The light microscope images are representative from five animals in each genotype. **b** Size of hepatocytes was not significantly different between $Hif1a^{+/+}$ and $Hif1a^{+/-}$ mice
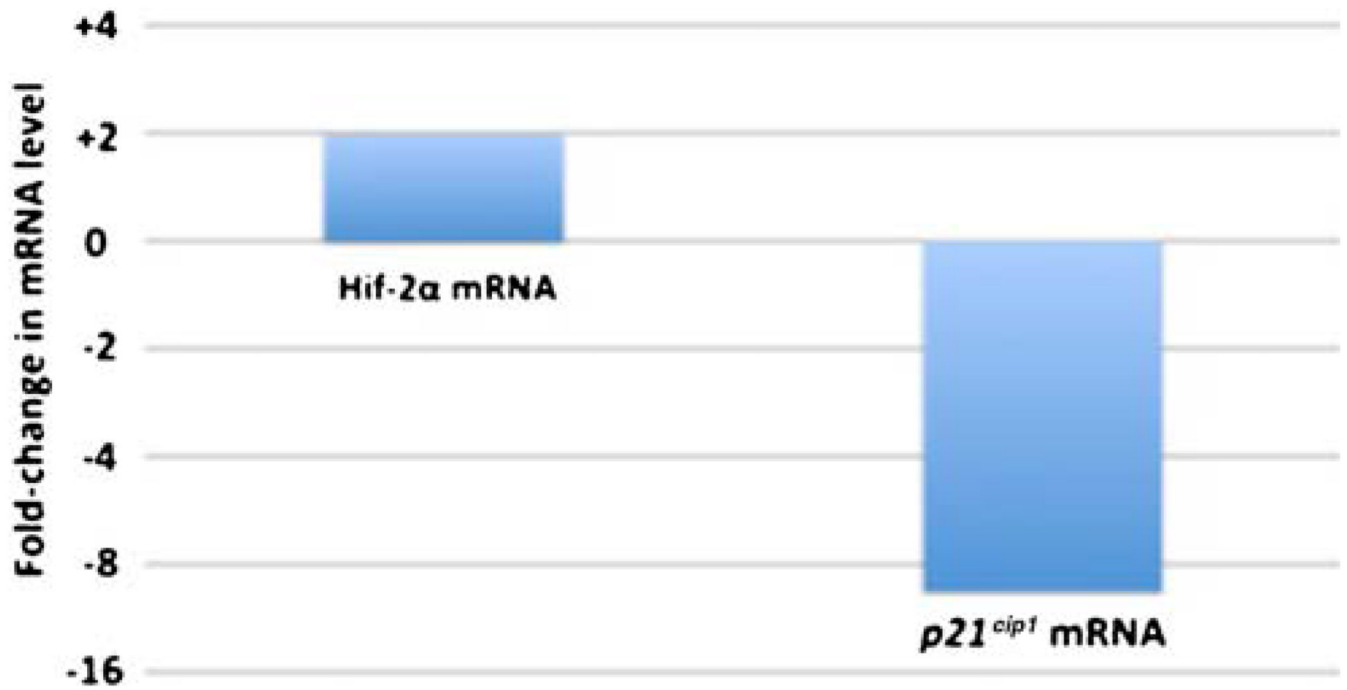
Fig. 2.
Analysis of gene expression in livers of $Hif1a^{+/+}$ and $Hif1a^{+/-}$ mice using real-time PCR. Quantitative real-time RT-PCR was performed using specific primers for HIF-2α and p21^{Cip1} mRNA. Fold-change in mRNA levels for HIF-2α and $p21^{Cip1}$ in $Hif1a^{+/-}$ mice compared to $Hif1a^{+/+}$ mice is depicted

Table 1

Clinical characteristics and organ volumes of study participants

	Chuvash polycythemia *N*=30	Controls *N*=30	*P*
Clinical characteristics			
Age (years)	47±8	47±8	–
Female sex	21 (70%)	21 (70%)	–
Weight (kg)	59.7±8.4	65.7±12.0	0.024
Height (cm)	162±6	163±9	0.315
Body mass index (kg/m^2)	22.8±2.9	24.6±4.2	0.040
Systolic blood pressure (mm Hg)	116±17	127±18	0.002
Diastolic blood pressure (mm Hg)	78±14	82±12	0.107
Hemoglobin (g/dL)	16.9±3.5	12.4±1.5	<0.0001
Organ volume			
Liver volume (cm^3)	1520±329	1369±303	0.041
Spleen volume (cm^3)	363±229	171±63	0.0002
Kidneys volume (cm^3)[a]	319±77	289±65	0.072
Organ volume to body mass ratio			
Liver (100× cm^3/kg)	2.57±0.54	2.09±0.29	0.0006
Spleen (100×cm^3/kg)	0.59±0.30	0.26±0.08	0.0001
Kidneys[a] (100×cm^3/kg)	0.54±0.14	0.45±0.09	0.005

Results in mean±SD or number of subjects (%). Comparison by the paired *t* test

[a]Combined volume of right and left kidneys

Table 2

Multiple linear regression analyses of organ volume in Chuvash polycythemia patients and controls

	Body weight increase of 10 kg	Male vs. female sex	Age increase of 10 years	Chuvash polycythemia vs. control
Liver (*N=62*)				
Estimated change in volume (cm^3)	166	253	−70	215
95% confidence interval	107–225	131–376	−145 to 5	104–327
P	<0.0001	0.001	0.068	0.0003
Spleen (*N=62*)				
Estimated change in volume (cm^3)	41	12	−9	172
95% confidence interval	15–66	−67 to 42	−41 to 23	123–221
P	0.003	0.657	0.579	<0.0001
Kidneys (*N=62*)				
Estimated change in volume (cm^3)	24	62	−15	30
95% confidence interval	10–38	32–91	−32 to 3	4–56
P	0.001	0.0001	0.095	0.026

Two additional controls added compared to Table 1

Table 3

Organ to body mass ratios of *Hif1a*$^{+/-}$ and *Hif1a*$^{+/+}$ mice

	Hif1a$^{+/-}$ (*N=7*)	*Hif1a*$^{+/+}$ (*N=5*)	*P*
Liver	5.27 (4.19–5.94)	3.51 (3.16–4.12)	0.004
Spleen	0.43 (0.17–0.79)	0.24 (0.12–0.34)	0.167
Kidneys[a]	1.69 (1.17–2.18)	1.20 (1.03–2.29)	0.372

Results shown are median (range). Statistical analysis was performed by the Kruskal–Wallis test

[a]Combined mass of right and left kidney