

- domain of NADH-cytochrome B5 reductase in an African-American family with type I congenital methemoglobinemia. *Blood* 1996;87:2993-9.
4. Chen L, Segal DM, Mash DC. Semi-quantitative reverse-transcriptase polymerase chain reaction: an approach for the measurement of target gene expression in human brain. *Brain Res Brain Res Protoc* 1999;4:132-9.
 5. Liu E, Jelinek J, Pastore YD, Guan Y, Prchal JF, Prchal JT. Discrimination of polycythemia and thrombocytosis by novel, simple, accurate clonality assays and comparison with PRV-1 expression and BFU-E response to erythropoietin. *Blood* 2003;101:3294-301.
 6. Dekker J, Eppink MH, van Zwieten R, de Rijk T, Remacha AF, Law LK, et al. Seven new mutations in the nicotinamide adenine dinucleotide reduced-cytochrome b(5) reductase gene leading to methemoglobinemia type I. *Blood* 2001;97:1106-14.
 7. Vieira LM, Kaplan JC, Kahn A, Leroux A. Four new mutations in the NADH-cytochrome b5 reductase gene from patients with recessive congenital methemoglobinemia type II. *Blood* 1995;85:2254-62.
 8. Holbrook JA, Neu-Yilik G, Hentze MW, Kulozik AE. Nonsense-mediated decay approaches the clinic. *Nat Genet* 2004;36:801-8.
 9. Cohen RJ, Sachs JR, Wicker DJ, Conrad ME. Methemoglobinemia provoked by malarial chemoprophylaxis in Vietnam. *N Engl J Med* 1963;279:1127-31.

Red Cell Disorders

Low frequency of *VHL* gene mutations in young individuals with polycythemia and high serum erythropoietin

In one out of six young individuals with polycythemia and high erythropoietin levels we found a heterozygous *VHL* gene mutation (430G→A; Gly144Arg). The man's unaffected mother and sister carry the same mutation. No other *VHL* genomic or expression alterations were found. In one other patient different genetic conditions were found.

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The molecular basis of congenital and familial polycythemia are largely unknown. Chuvash polycythemia (CP) is an autosomal recessive polycythemia with increased serum erythropoietin (Epo) caused by bi-allelic mutations of the von Hippel-Lindau (*VHL*) gene.¹ *VHL* mutations are also responsible for von Hippel-Lindau disease, an autosomal dominant syndrome predisposing to the development of multiple tumors.

Since 50% of sporadic cases of congenital CP with high serum Epo are attributed to bi-allelic *VHL* gene mutations,² we searched for *VHL* gene mutations in 4 previously described children with congenital polycythemia and unexplained high levels of serum Epo³ and in 2 other individuals with a similar phenotype. While in 5 of the cases polycythemia appeared to be congenital, in 1 case it was not possible to date the age of onset of the disease precisely. All patients were Italian from the Venetian region; none of them was of Chuvashian origin. None had splenomegaly or detectable causes of secondary erythrocytosis. In one patient neurofibromatosis type 2 was found in association with the polycythemia. The main clinical and molecular data of our patients are summarized in Table 1.

Serum Epo was measured with a commercially available immunoassay kit (Erythropoietin, Nicols Institute Diagnostics, CA, USA). We carried out the burst-forming unit-erythroid colony assay (EEC) on peripheral blood

mononuclear cells using standard methods.¹

Genomic DNA was extracted from peripheral blood leukocytes by conventional methods after signed informed consent, according to the Declaration of Helsinki. Mutation analysis was conducted on the entire coding sequence and intron-exon boundaries of the *VHL* gene by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis, followed by direct sequencing of the amplicons, as previously reported.⁴ Total cytoplasmic RNA was extracted from peripheral blood with RNazol (RNA-Bee, Tel-Test, Inc.) and retro-transcribed by random-priming.

The *VHL*-specific cDNA was amplified with primers 1CF (5'-GTGCTGCGCTCGGTGAAGCTC-3') and 3R (5'-CAAGACTCATCAGTACCATCAAAGCTG-3')⁵ which obtained amplification of two different isoforms, due to the normal alternative splicing of exon 2. The *VHL* isoform, including exon 2 was then sequenced with primers 2Fi (5'agg tca cct ttg gct ctt cag a 3') and 3R.

One out of six patients was heterozygous for a missense mutation involving the *VHL* amino acid residue in position 144 (430 G→A; Gly144Arg); molecular analysis of the mRNA confirmed the expression of the transcript from both *VHL* alleles. The same mutation was present in the man's 49 year-old mother as well as in one of his sisters (30 years old); neither had signs of polycythemia. A complete clinical work-up (ophthalmological and audiological evaluations, brain, spinal and abdominal CT scan/MRI and blood and urine measurement of catecholamines) failed to identify possible undetected *VHL*-related manifestations. Molecular analysis of the *VHL* gene in the other members of the proband's family was negative. The *VHL* mutation reported here was not detected in over 200 unrelated cases.

CP has been demonstrated to be associated with homozygosity for the 598C→T *VHL* mutation.⁶

Some non-Chuvash individuals with polycythemia and bi-allelic homozygous⁷ or compound heterozygous^{2,7,8} *VHL* gene mutations have also been described.

None of the individuals we have tested carried mutations on both *VHL* alleles and we have not detected the 598C→T mutation reported as the most important cause of congenital polycythemia with inappropriately high Epo serum levels.⁸ The *VHL* alteration found in our patient is a new mutation, never described in *VHL* disease or in patients with polycythemia, although *VHL* codon 144 has been previously found to be altered by missense mutations in patients with *VHL*-related tumors (www.umd.be:2020/ and HGMD).

The Gly144Arg mutation does not seem to cause *VHL* disease in the patient and his relatives. While the proband has congenital erythrocytosis, both his mother and sister carrying the mutation show no signs of polycythemia, and hence the mutation itself does not correlate with either phenotype. No other *VHL* mutation was detected at the DNA level; the transcripts of both *VHL* wildtype and mutated allele were documented at mRNA level. A similar situation has been previously observed (Table 2), although mRNA has not always been studied.

Since CP is considered an autosomal recessive condition, we hypothesize that when the heterozygous *VHL* mutation is present without other structural or transcriptional alterations, other as yet unknown factors might contribute to determining the polycythemia phenotype. However, the molecular mechanism of erythrocytosis in these cases remains to be elucidated.

It is worth noting that we found the association of

Table 1. Main clinical and laboratory characteristics of our patients.

Case (sex)	Age* (years, months)	Follow up duration (years)	RBC volume (mL/kg)	Hb (g/L)	HCT (%)	WBC ($\times 10^9/L$)	Platelets ($\times 10^9/L$)	EEC cultures	EPO (U/mL) N.V. 7-22	Associated clinical features	VHL Genetic
BD (M)	13y	17	55.8 (NV 25-30)	180 (NV 13-16)	56 (NV 37-46)	10.9	535	normal	207.3	none	Exon 2 VHL 430 G→A; G144R
RM (F)	3y 6m	16 (NV ~25)	49.3 (NV 112-143)	194 (NV 34-40)	65	5.98	290	not performed	31.7	Multiple cerebral ischemic lesions NF2	normal
DGL (F)*	3 y 8 m	16 (NV ~25)	67.1	167 (NV 112-143)	56.8 (NV 34-40)	6.2	307	normal	128	headache, dizziness	normal
DGG (M)*	4 m	13	#	156 (NV 9.5-12.5)	57.8 (NV 29-38)	8.0	363	normal	>200	none	normal
MA (M)	23 y	16	53 (NV <36)	173 (NV 131-163)	50.1 (NV 39-49)	6.2	840	normal	51	Budd-Chiari syndrome	normal
LN (F)	26 y	7	32§ (NV <32)	158 (NV 115-147)	48.2 (NV 34-45)	6.7	619	not performed	35	none	normal

*Age at first observation; NV: expected normal values for age (from Price DC, Ries C, in *Nuclear Medicine in Clinical Pediatrics 1975* and Dallman PR, in *Pediatrics, 1977*); RBC: red blood cell; Hb: hemoglobin; Hct: hematocrit; EPO: erythropoietin; NF2: neurofibromatosis type 2; *patients DGL and DGG are siblings; †not performed because of the young age and the reluctance to use radioactive substances.

Table 2. Heterozygous VHL mutations in individuals with erythrocytosis reported in the literature.

Individuals (sex)	VHL genotype	wt mRNA expression	Associated clinical features	Carrier family members	Ethnicity	Ref.
2 (M, F) siblings	376G→T / wt	Normal	F: renal subcapsular hemangioma M: none	Father (asymptomatic)	Ukrainian	2
1 (M)	598C→T / wt	Unknown	None	Mother and son (asymptomatic)	English	7
1 (F)	598C→T / wt	Normal	None	?	German	9
1 (F)	311G→T / wt	Normal	None	?	German (?)	9
1 (F)	523A→G / wt	Normal	Ataxia-tealeangectasia	Father (asymptomatic)	Portuguese	10
1 (M)	430G→A / wt	Normal	None	Mother and sister (asymptomatic)	Italian	Present paper

wt: wild type.

polycythemia with neurofibromatosis type 2. In this case Epo might be stimulated through other still unknown mechanisms.

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References

- Ang SO, Hirota K, Gordeuk VR, Jelinke J, Guan Y, Liu E, et al. Disruption of oxygen homeostasis underlies congenital Chuvash polycythemia. *Nature Genetics* 2002;32:614-21.
- Pastore Y, Jelinke J, Ang S, Guan Y, Liu E, Jedlickova K, et al. Mutations in the VHL gene in sporadic apparently congenital polycythemia. *Blood* 2003;101:1591-5.
- Randi ML, Mares M, Putti MC, Zanescio L, Girolami A. Clinical significance of serum erythropoietin in the diagnosis of polycythemia in pediatric age. 24th Congress Int Soc Haematol, London UK, 1992. p. 298.
- Murgia A, Martella M, Vinanzi C, Polli R, Perilongo G, Opocher G. Somatic mosaicism in von Hippel-Lindau disease. *Hum Mutat* 2000;15:114.
- Crossey PA, Richards EM, Foster K, Green JS, Prowse A, Latif F, et al. Identification of intragenic mutations in the von Hippel-Lindau disease tumor-suppressor gene and correlation with disease phenotype. *Hum Mol Genet* 1994;3:1303-8.
- Sergueeva A, Gordeuk V, Tokarev Y, Sokol L, Prchal JF, Prchal JT. Congenital polycythemia in Chuvashia. *Blood* 1997;89:2148-54.
- Percy MJ, McMullin MF, Potter M, Treacy M, Watson WH, Lappin TRJ. Chuvash-type congenital polycythemia in 4 fami-

- lies of Asian and Western European ancestry. *Blood* 2003; 102:1097-9.
8. Pastore Y, Jedlickova K, Guan Y, Liu E, Fahner J, Hasle H, et al. Mutations of von Hippel-Lindau tumor-suppressor gene and congenital polycythemia. *Am J Hum Genet* 2003;73:412-9.
 9. Cario H, Schwarz K, Jorch N, Kyank U, Petrides PE, Schneider DT, et al. Mutations in the von Hippel-Lindau (VHL) tumor suppressor gene and VHL-haplotype analysis in patients with presumable congenital erythrocytosis. *Haematologica* 2005; 90:19-24.
 10. Bento MC, Ko-Tung C, Guan Y, Liu E, Caldas G, Gatti RA, et al. Congenital polycythemia with homozygous mutations of von Hippel-Lindau gene: five new Caucasian patients. *Haematologica* 2005;90:128-9.

Myelodysplastic Syndromes

Lack of mutations of the human telomerase RNA gene (hTERC) in myelodysplastic syndrome

Myelodysplastic syndrome (MDS), considered a pre-leukemic state, has recently been categorized as a subset of bone marrow failure syndromes. Unlike other subtypes of bone marrow failure syndromes, such as aplastic anemia or dyskeratosis congenita,¹ little is known about genetic alterations of human telomerase in MDS, despite the fact that immune cells from patients with MDS frequently exhibit telomere attrition.

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Human telomerase RNA (hTERC) is an essential component of the telomerase ribonucleoprotein complex, and mutations in hTERC can result in haploid insufficiency, reducing telomerase activity,² leading to premature telomere shortening. Identification of mutations of hTERC in bone marrow failure syndromes, including myelodysplastic syndrome (MDS), may provide insights into the underlying molecular causes of these syndromes.

In the present study, we investigated mutations of the hTERC gene (*NT 005612.14*) using polymerase chain reaction-direct sequencing in 42 marrow samples from 35 consecutive MDS patients (34 to 80 years old); 19 had refractory anemia (RA), 14 had RA with excess blasts (RAEB), and two patients had RAEB in transformation. Seven RAEB patients were also studied at the time their disease transformed into acute myeloid leukemia. Blood samples were also obtained from 134 healthy volunteers (4 to 90 years old). All samples were collected from Japanese patients and healthy volunteers after obtaining informed consent. Telomere length and telomerase activity were measured as previously described in mononuclear cells.³

We selected seven hTERC loci; C98T, the template region G58A, pseudoknot domain C72T and Δ 110-113, CR4-CD5 domain G305A and G322A, and Box H/ACA domain G450A, to identify possible mutations of the hTERC gene. We also examined polymorphisms at 514. Direct sequencing showed no heterozygous hTERC mutations of these loci in 42 MDS samples and 134 healthy volunteers, although MDS patients had variable telomere lengths (short in 27%, normal in 69%, and long in 5% compared to normal volunteers) with low telomerase activity. We did not find allelic variations at the 514 locus in healthy populations: AA genotype (MDS 11.1% versus control 11.9%), AG genotype (MDS 55.6% versus control 51.4%), and GG genotype (MDS 33.3% versus control 36.7%) and no deviation was notable in MDS patients.

hTERC mutations at certain loci affect telomerase activity, and most MDS patients show normal to low levels of telomerase activity; nevertheless, cells from some MDS patients have telomere attrition. In one study it was reported that, out of 55 MDS patients, two black patients had a G58A change and one other patient had a G322A substitution.⁴ More recently a black MDS patient with G58A was also reported.⁵ Since the G58A substitution seems to be common in the black population (5/24 normal subjects)⁵ and no G58A mutations in the hTERC gene were detected among the normal Japanese population or in the MDS patients in our study, mutations in the hTERC gene are unlikely to be related to telomere changes observed in most MDS patients. Since some MDS patients have shortened telomeres and also low telomerase activity, it remains possible that the dysfunction of telomere regulation in MDS patients may be caused by alterations in other proteins that interact with telomerase or in the catalytic component (hTERT) itself.

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References

1. Fogarty PF, Yamaguchi H, Wiestner A, Baerlocher GM, Sloand E, Zeng WS, et al. Late presentation of dyskeratosis congenita as apparently acquired aplastic anaemia due to mutations in telomerase RNA. *Lancet* 2003;362:1628-30.
2. Ly H, Blackburn EH, Parslow TG. Comprehensive structure-function analysis of the core domain of human telomerase RNA. *Mol Cell Biol* 2003;23:6849-56.
3. Iwama H, Ohyashiki K, Ohyashiki JH, Hayashi S, Yahata N, Ando K, et al. Telomeric length and telomerase activity vary with age in peripheral blood cells obtained from normal individuals. *Hum Genet* 1998;102:397-402.
4. Yamaguchi H, Baerlocher GM, Lansdorp PM, Chanock SJ, Nunez O, Sloand E, et al. Mutations of the human telomerase RNA gene (TERC) in aplastic anemia and myelodysplastic syndrome. *Blood* 2003;102:916-8.
5. Wilson DB, Ivanovich J, Whelan A, Goodfellow PJ, Bessler M. Human telomerase RNA mutations and bone marrow failure. *Lancet* 2003;361:1993-4.

Myelodysplastic Syndromes

Secondary myelodysplastic syndromes following treatment with azathioprine are associated with aberrations of chromosome 7

We report 14 cases of secondary myelodysplastic syndromes (sMDS) following treatment with azathioprine for non-malignant disorders. Long-term treatment with azathioprine seems to be associated with an increased risk of MDS and subsequent leukemic transformation.

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