Chuvash-type congenital polycythemia in 4 families of Asian and Western European ancestry

Short title: Chuvash-type congenital polycythemia

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We wish to thank all the clinicians that have referred erythrocytosis patients from UK and Ireland to our registry and provided patient samples.
Abstract

The Chuvash form of polycythemia is an autosomal recessive disorder common to a large number of families in central Russia. Affected individuals have been reported to be homozygous for an Arg200Trp mutation in the von Hippel-Lindau (VHL) gene. We have screened 78 patients with erythrocytosis and found eight of Bangladeshi and Pakistani origin to be homozygous for the Arg200Trp mutation and another of English descent to be heterozygous. Five of these patients have elevated serum erythropoietin (Epo) levels, while the other four have Epo values in the normal range. The heterozygous patient does not fulfil the Chuvash criterion for homozygosity of Arg200Trp mutation and consequently may harbor a further, as yet uncharacterized mutation. This mutation has a wider geographical distribution than originally presumed and haplotype analysis suggests a common origin of the Arg200Trp mutation in the four families but it still remains to be established if it has arisen independently of the Chuvash population.

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Introduction

The autosomal recessive disorder known as the Chuvash form of familial polycythemia affects over 100 individuals from more than 80 families in the mid-Volga Region of European Russia.\(^1\) It is characterized by high hemoglobin levels, usually more than 20 g/dL, and normal to raised plasma erythropoietin (Epo). A genome-wide screen indicated the location of a candidate gene on chromosome 3 and sequencing the von Hippel-Lindau gene (VHL) detected a homozygous mutation (Arg200Trp) in Chuvash polycythemic individuals.\(^2\) Subsequently this and three other VHL mutations (Asp126Tyr, Val130Ile, Pro192Ala) were detected in five polycythemic children of Dutch-Italian, English and Russian origins.\(^3,4\)

Epo production is tightly regulated by a negative feedback loop\(^5\) involving the transcription complex HIF-1.\(^6\) This complex is composed of two subunits, HIF-1\(\alpha\) and the constitutively stable HIF-1\(\beta\) (also known as ARNT).\(^7\) Under normoxia the level of HIF-1\(\alpha\) is maintained at a low level as it binds to the VHL protein, becomes ubiquitinated and is targeted by the elongin C complex for degradation by the proteasome.\(^8\) A point mutation in the VHL gene is known to disrupt its association with HIF-1\(\alpha\) and increase Epo production.\(^9\) Thus we decided to screen for VHL mutations in a group of erythrocytosis patients with normal to raised Epo levels, who were negative for Epo receptor (EpoR) mutations.

Study Design

Patients and Case History

Idiopathic erythrocytosis patients from the UK and Ireland with elevated hemoglobin (Hb) (greater than 18 g/dL in males and 16.5 g/dL in females), raised packed cell
volume (PCV) (above 0.51 in males and 0.48 in females), with no splenomegaly and the absence of known secondary causes were recruited to our registry. Epo levels were measured locally and the normal range (NR) for each assay is given.

Figure 1A.

FAMILY A

Figure 1 A-D. Pedigrees of the four families with the C598T mutation. Circles denote female family members, squares denote male family members and symbols with a diagonal line indicate deceased family members. Black symbols indicate individuals with erythrocytosis and shaded symbols non-erythrocytosis carriers for the C598T mutation. The genotype of individuals is indicated by wt (normal) and C598T for mutant. Hemoglobin (g/dL), PCV, red blood cell count (RBC) and Epo (mU/mL) values are given when known.

Family A. A Bengali speaking family from Bangladesh (Figure 1A) was investigated following the identification of the propositus (A1) who presented at the age of 21 with peripheral neuropathy. A CT scan of his head was normal. He had a Hb of 22.6 g/dL, PCV of 0.67, normal P50 value and an Epo level of 48 mU/mL (NR 3.7-15.2 mU/mL). Three other siblings (A2, A3 and A4) with normal Epo levels (7.2-10.1...
mU/mL, NR 3.7-15.2 mU/mL) had elevated hemoglobin. There is no consanguinity in the family. None of the patients have a history of cerebrovascular complications.

Family B. A 32 year old Punjabi speaking female (B1, Figure 1B) from the Toba Tek Singh district of Pakistan attending an infertility clinic was found to have a hemoglobin of 20.7 g/dL and PCV of 0.66 with a normal P50 value. A CT scan following a stroke showed a cerebral infarct but no evidence of cerebrovascular malformation. Her twenty year old sister (B2) was admitted to hospital with a large left parietal intracerebral hematoma. Cerebral angiograms and MRI scans did not indicate any cerebrovascular abnormalities. B2’s red cell count was elevated at 6 x 10^{12}/L with a hemoglobin of 13.3 g/dL and PCV of 0.43. A second sibling (B3) presented with hemoglobin of 16.7 g/dL and PCV of 0.67 with no history of stroke and so had no cerebrovascular investigation. Both B2 and B3 were iron deficient. Epo levels for B1-B3 were 369 mU/ml, 128 mU/ml and 105 mU/ml (NR 2.5-10.5 mU/ml)
respectively. The older members of the next generation are normal and there is no history of erythrocytosis in both maternal and paternal relatives although both parents are first cousins.

**Figure 1C**

![Family Tree](image)

**FAMILY C**

*Patient C1.* An eight year old Urdu speaking boy from the Punjab region of Pakistan (Figure 1C) presented with a hemoglobin of 24.4 g/dL and PCV of 0.61. His Epo level was 96 mU/mL (NR 9.1-30.8 mU/ml). Both parents are cousins and there is a family history of infant mortality with one baby born dead, three dying within a few days of birth of unclear causes and another sibling who died at the age of six with a cerebrovascular accident. There is one normal surviving female now eight years old.
Patient D1. A white Caucasian male of English ancestry was diagnosed with erythrocytosis at the age of 34 years without a family history (Figure 1D). At presentation his hemoglobin was 21.4 g/dL and PCV was 0.67. His Epo level was 26.1 mU/mL (NR 8-28 mU/mL). All other parameters were normal. One year post diagnosis D1 experienced a thrombosis in the left leg.

Sequencing the VHL gene

Genomic DNA was isolated from peripheral blood using a Nucleon BACC 1 DNA extraction kit (Nucleon Biosciences, Manchester, UK). PCR was performed in 100 µL reaction containing 10 % DMSO, 1.5 mmol/L MgCl₂, 200 µmol/L dNTP, 0.5 µmol/L each of forward and reverse primer, 2.5 units of Thermo-Start DNA Polymerase (ABgene, Epsom, Surrey, UK) and 100 ng genomic DNA, in 1x Thermo-Start Standard Buffer provided by the manufacturer. The following primer sets were used: VHL F1 5’-AGCGCGTTCCATCCTCTAC-3’ and VHL R1 5’-GCTTCAGACCGTGCTATCGT-3’ for exon 1;
VHL F2 5’-GAGGTTTCACCACGTAGCC-3’ and VHL R2 5’-
AGCCCAAAGTCTTTTGAGA-3’ for exon 2; VHL F3 5’-
CAGAGGCAAGCAACCACCATGA-3’ and VHL R3 5’-
AAGGAAGGACCAGTCCTGT-3’ for exon 3. Amplification was performed with an initial heat activation step of 15 min at 95°C followed by 35 cycles for 1 min at 95°C, 52°C for 1 min and 72°C for 1 min in the GeneAmp PCR system 2700 (Applied Biosystems, Warrington, UK). PCR products were purified using Concert Rapid PCR Purification System (Life Technologies, Paisley, UK) and were sequenced using ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit Version 3 on ABI 3100 DNA Genetic Analyzer (Applied Biosystems).

Mutation screen for C598T base change.
The C598T mutation destroys the restriction site for BsrB1 enzyme and 16 µL of PCR amplified exon 3 were digested with 10 U of BsrB1 (New England Biolabs, Hitchin, UK) for 2 hours at 37°C and visualized by agarose electrophoresis.

Haplotype analysis.
Single nucleotide polymorphisms (SNPs) located in introns 1 and 2 of the VHL gene, 3 kb 5’, 3 kb and 8 kb 3’ to the VHL gene, were selected from Chromosome 3 reference contig NT_005927 (NCBI). The following primer sets were used:
rs776517 F 5’-GCCACCCCCCTTCTCTTAAAT-3’ and rs776517 R 5’-
CCCCAAAGTAGGACTTCTGTTA-3’; for SNP at 10,109,893 primers rs775617F/R were used; rs374645 F 5’-TTTACATTTCTTAAAAATTTCCCATCA-3’ and rs374645 R 5’-TCAGTCCTCACAACAGATCCA-3’; rs260005 F 5’-
CCCCAAATACATGGGTGTTCA-3’ and rs260005 R 5’-
CAACGACTACCACCAGCAGA-3’; rs166538 F 5’-
CAAGGTGGTGAACCTCTGTC-3’ and rs166538 R 5’-
TTGGGCAATCTCCCATACAT-3’; rs8952 F 5’-TTGATCTTGAGCGTTTTGGA-3’ and rs458952 R 5’-GTATTCTGACCCCAGGCTCTC-3’. Amplification was as described above with an annealing temperature of 55°C and PCR products were sequenced.

Results and Discussion

A group of 46 patients with raised and normal Epo levels were chosen from our registry of erythrocytosis patients. Sequencing all three exons of the VHL gene detected a homozygous C to T transition at nucleotide 598 in 4 siblings of family A (Figure 2A), 3 siblings from Family B and patient C1. A ninth patient (D1) was found to be heterozygous for this mutation (Figure 2C). The C598T mutation destroys the restriction site for BsrB1 enzyme and a further group of 32 erythrocytosis patients with low to normal Epo levels were screened for the C598T mutation and found to be negative.

The C598T mutation predicts an amino acid change of arginine to tryptophan at codon 200 (Arg200Trp), which is located outside the seven-stranded β domain of pVHL that binds HIF-1α (residues 63 to 154), but lies within the α helix (residues 193-204).10 Although this helix does not bind directly to HIF-1α it may contribute to the association of VHL and HIF-1α.10 The Arg200Trp mutation affects the affinity of pVHL for HIF-1α thereby prolonging the stability of HIF-1α and upregulating Epo production.11
Figure 2. Detection of C598T mutation. Sequencing of exon 3 of the VHL gene detected a homozygous base change of C to T at nucleotide 598 in patients A1-A3, B1-B3 and C1 (Figure 2A) when compared to the normal sequence (Figure 2B). One patient, D1, was heterozygous for this base change (Figure 2B). The position of the mutation is indicated by an arrow. The C598T mutation destroys the restriction site for BsrB1 enzyme and the normal exon 3 PCR product is restricted into 2 fragments of 330 and 132 bp but in the presence of the C598T mutation only a 462 bp fragment is obtained. Screening of family members of C1 indicated that his mother was heterozygous and his sibling was normal (Figure 2D). The father of D1 is normal but his mother and son are heterozygous for the C598T (Figure 2E).

M: 100 bp DNA ladder; MC1: mother of patient C1; C1: patient C1; SC1: sibling of C1; FD1: father of patient D1; MD1: mother of patient D1; D1: patient D1; SD1: son of C1;

All patients reported here, except D1, fulfil the Chuvash polycythemia criteria clinically, with a recessive mode of inheritance. A further unidentified mutation may contribute to D1’s erythrocytosis. Although the mother and the twenty year old son of
D1 are heterozygous for the C598T mutation they have not been diagnosed with erythrocytosis, and D1’s son has a hemoglobin of 17.3 g/dL, at the upper end of the normal range but he is a smoker. In common with previous reports we have found the Arg 200Trp mutation is associated with both raised or inappropriately normal serum Epo values. 1-4, 11

The Arg200Trp mutation has been implicated as the cause of autosomal recessive Chuvash polycythemia2 and we have detected the same mutation in families of Bangladeshi, Pakistani and English origin. It appears that the C598T mutation has not arisen de novo in these patients as several members in families A and B possess the mutation and mothers from families A, C and D are heterozygous (Figure 2D and 2E). To complete the study analysis of the surviving parent of family B and patient C1’s father is required. To date the Arg200Trp mutation has been reported in Chuvashian2, Dutch-Italian, English, Russian and African-American individuals3,4, patients with VHL syndrome12,13 and clear cell carcinoma14,15 (http://www.umd.necker.fr:2005/), thus suggesting that this mutation has arisen independently in different ethnic groups or that it has a wider geographical distribution than initially presumed. To identify the origin of Arg200Trp mutation haplotype analysis has been performed with families A, B, C and D using SNPs located in introns 1 and 2 of the VHL gene, and 3 kb 5’, 3 kb and 8 kb 3’ to the VHL gene (Table 1).
Table 1. Allele frequencies of 6 dimorphic SNPs located within the VHL gene and flanking regions in normal individuals and subjects bearing the C598T base change.

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<th>Homozygotes (N=16)</th>
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rs number - reference number in SNPs data from NCBI
bp – location on Chromosome 3 reference contig NT_005927 (NCBI)
N – number of alleles

A common haplotype of GCTACA was found to be associated with the Arg200Trp mutation (Table 2) thus suggesting a founder mutation in our group of patients. A comprehensive haplotype analysis of individuals from Chuvashia and the other ethnic groups will establish if there was a common founder mutation that has been distributed by migration.
Table 2. Frequency of the founder haplotype (GCTACG) in normal 598C and 598T alleles

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References


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