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Genetic Adaptation to Extreme Hypoxia. Study of High-Altitude Pulmonary Edema in a Three Generations of Chinese Han Family

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Abstract

Organismal response to hypoxia is essential for critical regulation of erythropoiesis, other physiological functions and survival. There is an evidence of individual variation in response to hypoxia as some but not all of the affected individuals develop polycythemia, and or pulmonary and cerebral edema. A significant population difference in response to hypoxia exist as many Tibetans, Ethiopian and Andean natives developed an adaptive mechanisms to extreme hypoxia. A proportion of any non-adapted individuals exposed to high altitude develop pulmonary edema (HAPE), pulmonary hypertension, cerebral edema and extreme polycythemia. The isolation of causative gene(s) responsible for HAPE and other extreme hypoxia complications would provide a rational basis for specific targeted therapy of HAPE, allow its targeted prevention for at-risk populations, and clarification of its, and pathophysiology of other hypoxic maladaptations. As today, the only suggested linkage in unrelated individual with HAPE has been with endothelial nitric oxide synthase (eNOS) gene. Here we describe a family with multiple members affected with HAPE in three generations. Families with multiple affected members with HAPE have not been described. We first ruled out linkage of HAPE with eNOS gene. We then performed analysis of the whole genome using high-density SNP arrays (Affymetrix v5.0) and assuming a single gene causation of HAPE ruled out a linkage with 34 other candidate genes. Only HIF2A haplotype was shared by individuals who exhibit the HAPE phenotype, and the work on their possible causative role in HAPE is in progress. Clearly a small size of our family does not provide sufficient power for a conclusive analysis of linkage; we hope that collaboration with other investigators referring us more HAPE patients in effort to increase sample size would lead to identification of gene(s) responsible for HAPE and possibly other maladaptive hypoxic complications.

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Keywords

Hypoxia; Erythropoietin; High Altitude Pulmonary Edema (HAPE); Endothelial Nitric Oxide (eNOS); Hypoxia Inducible Factor (HIF)

INTRODUCTION

The response to hypoxia is crucial for multiple body functions, including erythropoiesis, energy metabolism, respiration, angiogenesis and other functions.[1] The transcription of as many as 3% of the genes in the genome is regulated by ubiquitously expressed transcription factor HIF-1, a master transcription factor discovered from studies of erythropoietin gene regulation.[2] In some tissues, HIF-1's homologue HIF-2 regulates distinct functions (for example renal erythropoietin gene is regulated by HIF-1 but hepatic and cerebral erythropoietin expression is regulated by HIF-2)[3] and both HIF-1 and HIF-2 are regulated not only by hypoxia but by other mechanisms including heat shock protein 9-(HSP 90 and RACK1) as well as oxygen radicals.[4,5]

Hypoxic preconditioning of mammalian cells leads to limited hypoxic response after re-oxygenation and is associated with protective response to ischemic damage.[6] Further, normoxic mice with prior hypoxic exposure have an augmented ventilatory response to subsequent acute hypoxia.[7] The current concept of hypoxia induced regulation is depicted in Figure 1.[8]

There is clear evidence of individual variation in response to hypoxia as witnessed by the fact that hypoxemia from pulmonary or heart disease, or from exposure to extreme high altitude, leads in some but not all of the affected individuals to an appropriate increase of red cell mass.[9] Further, athletes trained in hypobaric chambers have variable responses to improved athletic performance that correlates with commensurate increase of erythropoietin levels.[10,11] The individual variation of erythropoietic responses to a hypoxic stimulus suggests that there are likely genetic determinants including genetic polymorphisms underlying the erythropoietic response to hypoxia.

Similarly, there are significant population differences in response to hypoxia. Native Tibetan populations, highlanders in Ethiopia, and populations in the Andes appear to have developed an adaptive mechanism to protect against extreme hypoxia.[12] However, a proportion of non-adapted Caucasians and Han Chinese and other populations exposed to extreme hypoxia of high altitude tend to develop significant complications in extreme hypoxia which include high altitude pulmonary edema (HAPE), cerebral edema, pulmonary hypertension[13], and extreme polycythemia[14], these complications can be fatal.

In non-acclimatized individuals one of these complications, HAPE, can occur at as low as 2,500 meters. This often lethal complication occurs with what appears to be individual predisposition.[15,16] The only gene with suggested a linkage with HAPE is endothelial nitric oxide synthase (*eNOS*). Its polymorphisms in studies of unrelated individuals Japanese and Asian Indian ethnic subjects.[16,17] There are reports that carriers of the *27bp VNTR* of *eNOS* gene have lower nitric oxide plasma levels and decreased protein expression.[16,17] Lower *eNOS* mRNA and serum nitric oxide levels were found in individuals with the *−786C eNOS* variant, this association was strengthen by *in vitro* reporter gene assays.[21] Human *in vivo* studies suggested that subjects homozygous for the −786C allele have a decreased maximal forearm blood flow response to acetylcholine, a pharmacologic tool to evaluate nitric oxide production *in vivo*.[22,23]

However, to our knowledge, families with multiple affected members with HAPE have not been described. In this report, we describe one such family and demonstrate the lack of association of linkage with the *eNOS* gene. We also performed preliminary analysis of the whole genome using high-density SNP arrays, ruled out a linkage with other candidate genes; we believe extending these studies to other affected families or multiple individuals should lead to identification of gene(s) responsible for HAPE.

MATERIALS AND METHODS

DNA isolation

Blood samples were processed with the approval of Ethics Committee of Institute of High Altitude Medicine of Qinghai University at Xining, China. Genomic DNA was extracted from peripheral blood using the Gentra Puregene Blood Kit (Qiagen, Germantown, MA) following the manufacturer's instruction, and concentration determined using an ND-1000 spectrophotometer (Thermo Scientific, Nanodrop, Wilmington, DE). Quality and sizing of genomic DNA was determined by agarose gel electrophoresis.

Evaluation of *eNOS* gene polymorphism, its linkage to HAPE, and whole genome SNP study

We examined the *894G/T, −922A/G, −786T/C* polymorphisms and *27bp-VNTRs* in *eNOS* gene. The genotype of these polymorphisms were identified with a polymerase chain reaction (PCR) followed by sequencing of both strands. The primers applied to the current PCR experiments were as follows: *eNOS*894-F: 5'-AAGGCAGGAGACAGTGGATGGA-3', *eNOS*894-R: 5'-CCCAGTCAATCCCTTTGGTGCTCA-3' for 894G/T amplification; *eNOS*27-F: 5'-AGGCCCTATGGTAGTGCCTT-3', *eNOS*27-R: 5'-TCTCTTAGT GCTGTGGTCAC-3' for *27bp-VNTRs* amplification; 5UTR-F1: 5'-CTCTGAGCACA GCCCGTTCC-3', 5UTR-F2: 5'-CCCACCCCAACCTTATCCTC-3', 5UTR-R: 5'-ATG GCAGCCCACACTCTCAG-3' for −922A/G, −786T/ C polymorphisms detection.; The reactions were performed using standard protocol. The PCR product of *27bp-VNTRs* was detected by 3% agarose gel electrophoresis. Other products were electrophoresed on a 1% agarose gel and purified with QIAquick Gel Extraction Kit (Qiagen, Germantown, MA). Sequencing of the PCR products was done on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Affymetrix GenomeWide SNP 5.0 arrays were used following the protocol supplied by the manufacturer (Affymetrix, Santa Clara, CA). Briefly, 250 ng genomic DNA were digested for Nsp and Sty enzymes, followed by ligation-specific adaptors and amplified by PCR using the kit primer. The amplicons were purified and quantified. The products were fragmented and labeled, following by hybridization to the array chips at 48°C for 16–18 hours. Excess unhybridized products were washed, followed by scanning with a GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA). Genotypes were called using the Affymetrix BRLMM-p algorithm as implemented in the Genotyping Console software (Affymetrix, Santa Clara, CA). All samples had BRLMM call rates greater than the 95% cutoff.

We conducted a genome-wide scan of single nucleotide polymorphisms (SNPs) to determine whether we could exclude candidate genes associated with hypoxia sensing, assuming a single-gene dominant mode of inheritance. We examined patterns of haplotype inheritance using the pedigree analysis software package MERLIN Abecasis (www.sph.umich.edu/csg/abecasis/merlin). The most likely SNP haplotypes were estimated for 100KB regions spanning 35 candidate gene regions (Supplementary Table 1) by assessing informative meioses in the pedigree. We also used nonparametric linkage analysis to determine LOD

scores for each region (Kong and Cox 1997), although pedigree size limits the statistical power to detect linkage.

RESULTS

Clinical presentation and description of phenotype

An 11-year-old boy, ethnic Han Chinese, was brought by ambulance to the emergency department of Lhasa Hospital (altitude 3,648 m). As related by history, upon arrival to Lhasa airport he experienced severe headache, nausea, and breathlessness. A few hours latter, he rapidly developed tachypnea and labored breathing and expectorated foamy red-tinged sputum. He was taken to Lhasa hospital. Physical examination revealed a marked cyanosis, tachypnea, and tachycardia, slight right ventricular heave and a loud second heart (pulmonary) sound. Rales were present extending from lung bases to the middle lung fields; the arterial oxygen saturation (SaO_2) was 65%. The chest radiography showed moderate infiltrates in the both lung fields and slight prominence of the pulmonary artery and its branches, cardiac size was normal. He was treated with oxygen and pulmonary vasodilators. The clinical signs and symptoms rapidly improved, and SaO_2 was normalized. He was diagnosed with high-altitude pulmonary edema (HAPE).

The propositus was born and raised in Chendu (altitude 500 m), Sichuan province of China, and had experienced three previous episodes of HAPE while visiting his father in Lhasa, Tibet. After the propositus' hospital discharge and when fully recovered, his mean pulmonary arterial pressure measured by Doppler echocardiography after breathing 10% O_2 was increased from 15mmHg to 25mmHg consistent with pulmonary hypertension. His plasma concentration of nitrite and nitrate were markedly lower (12.93 μmol/L) when compared with age- and sexmatched mean controls (33.80 μmol/L).

Family Studies

The detailed interview revealed other family members with documented HAPE, all requiring hospitalizations (confirmed by review of medical records). These affected relatives included an 8-year-old first cousin (III-2), the boy's father (II-2), both his siblings (II-3 and II-5) and parental grandfather (I-1), all living in Lhasa while the members of the third generation and paternal grandmother (I-2) live in low altitude and visit Lhasa on rare occasions (Figure 2).

While the propositus had a typical presentation of recurrent HAPE [24,25] he also had a clear genetic susceptibility to HAPE as evidenced by multiple affected first-degree relatives. HAPE is a rare life-threatening condition characterized by marked pulmonary vasoconstriction, which normally occurs in healthy persons after rapid exposure to altitudes in excess of 2,500 m. above sea level. The pathogenesis of the recurrent HAPE and familial HAPE has not yet been delineated.

eNOS

In view of these reports, we analyzed the 4 polymorphisms of *eNOS* gene in our HAPE family and found that only the patient harbors the following *eNOS3* polymorphisms: *27bp-VNTRs* 4a/b, $T^{-786}\rightarrow C$, $A^{-922}\rightarrow G$. Thus linkage of HAPE with *eNOS* could not be established in this family using these particular polymorphisms. We then employed 3 microsatellites in the vicinity *eNOS3* gene (GATA45E12--Chromosome 7:162.33cM, D7S642--Chromosome 7: 162.62cM, GATA189C06--Chromosome 7:163.03cM) to analyze all family members and found no linkage of HAPE with *eNOS* (Figure 2. microsatellite in HAPE pedigree). Thus *eNOS* cannot account for the HAPE familial phenotype in this Chinese family.

Based on SNP analysis, the *eNOS* gene is not linked to the disease phenotype. Assuming a single gene causes HAPE, we ruled out linkage with 34 other candidate genes. Only HIF2A haplotype was shared by individuals who exhibit the HAPE phenotype (Supplementary Table 1). Therefore, this gene is not excluded based upon patterns of inheritance and will require further investigation. The LOD scores calculated for each of these regions are limited by the small pedigree size and are not reported here (Supplementary Table 1). Although this initial assessment does not produce enough evidence for linkage, the observed patterns provide insight for future analyses.

DISCUSSION

Clearly, the isolation of causative gene (s) responsible for HAPE would provide a rationale basis for specific targeted therapy and allow its targeted prevention for at-risk populations. Because of absence of linkage with the *eNOS* gene, we initiated a whole genome screen using Affymetrix SNP5.0 chips these analysis also ruled out linkage with *eNOS* gene. While the relatively small family size does not provide sufficient power for conclusive linkage analysis, these data can be utilized as a background for determining the region of the genome associated with HAPE and provide a basis for isolating the causative gene and elucidating the molecular basis and pathophysiology of HAPE. The low incidence of familial HAPE will require collaboration with other investigators with access to HAPE families, or HAPE affected individuals that should result in identification of causative gene(s) responsible for HAPE. When this is accomplished, this new knowledge should improve the prevention of HAPE by identification of individuals at risk, lead to development of HAPE targeted therapy, and clarification of HAPE pathophysiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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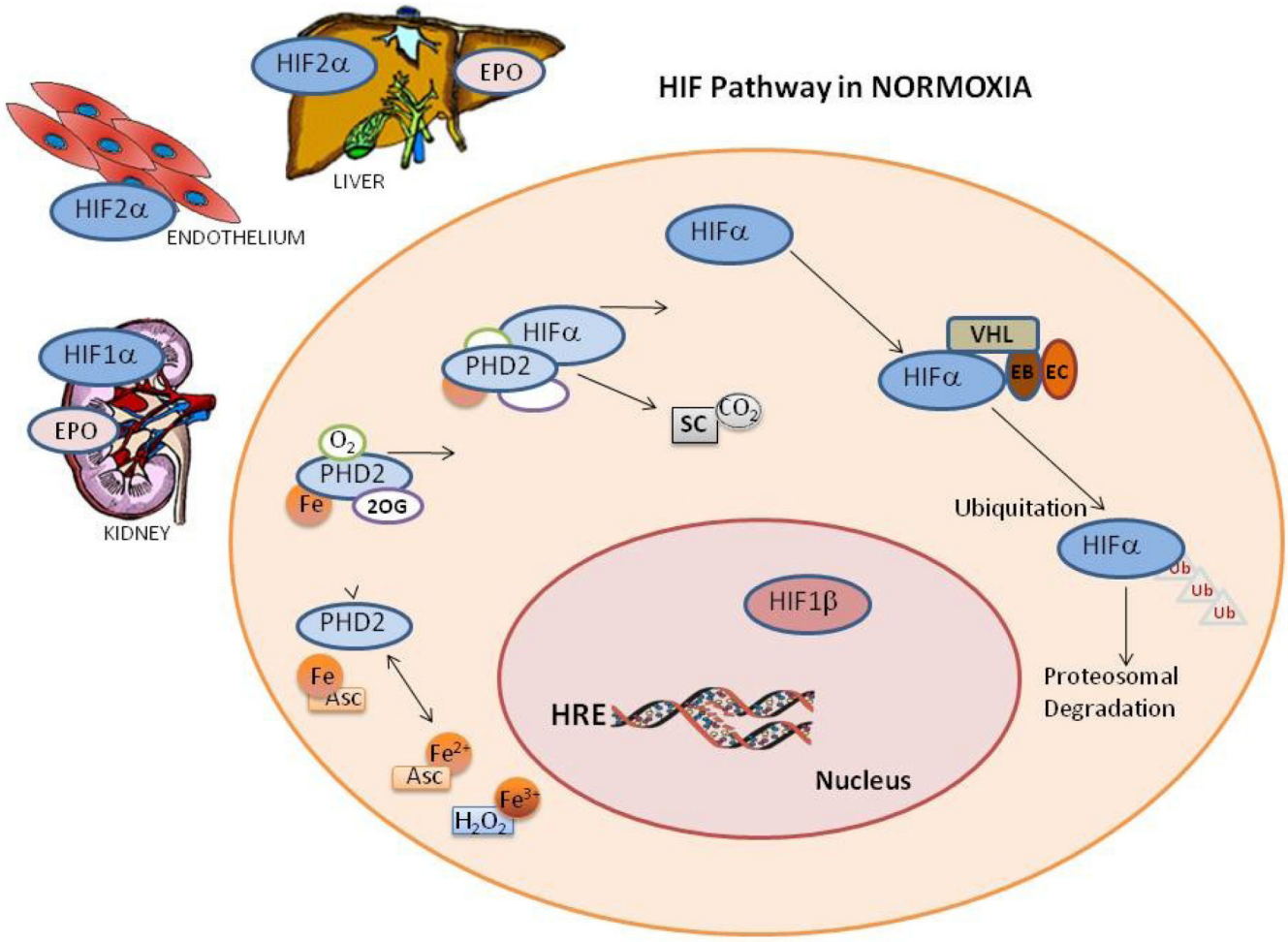

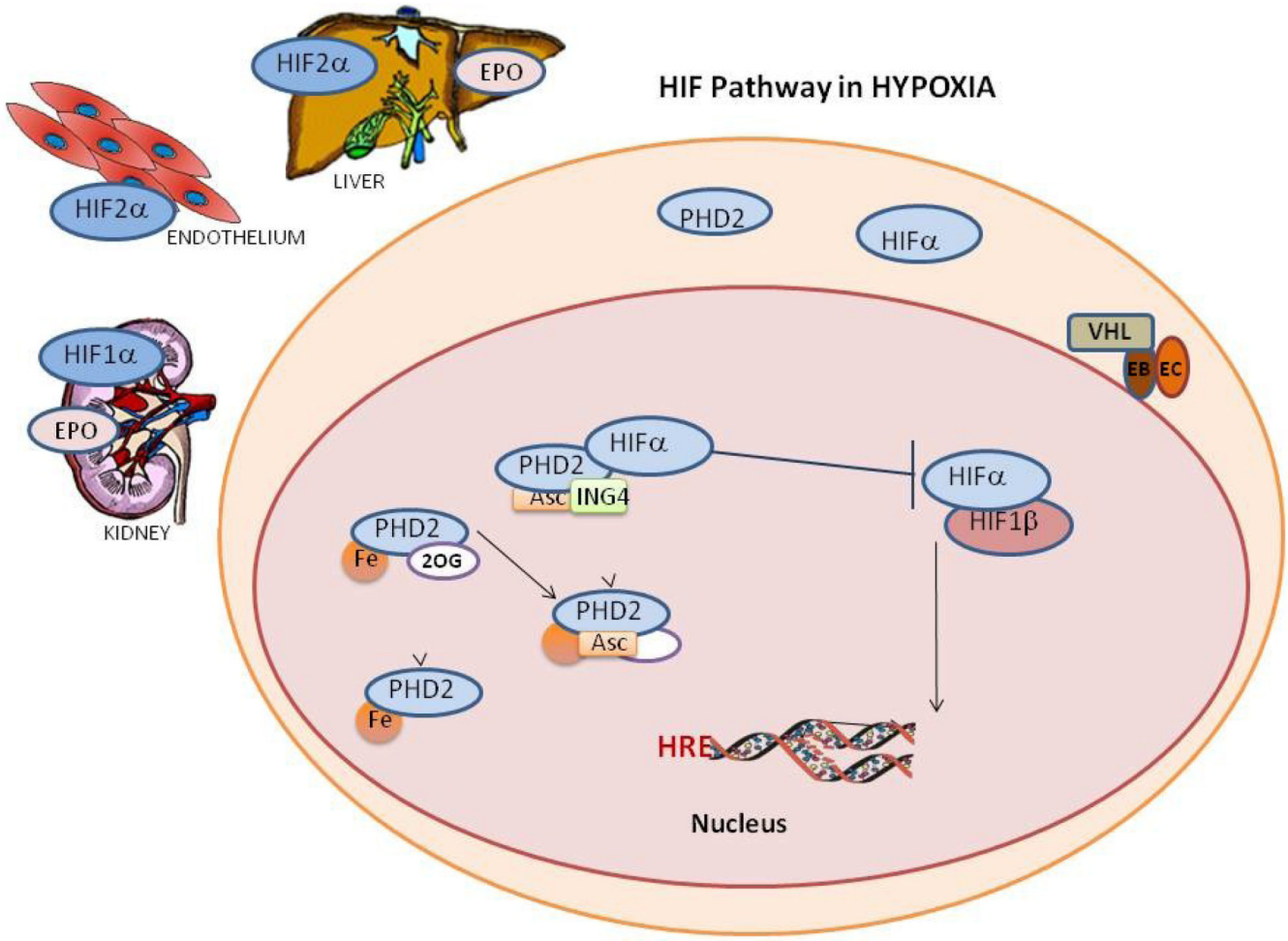

Figure 1. Hypoxia Inducible Factor (HIF) pathways

A. In normoxia HIF hydroxylation and degradation occurs in the cytoplasm which involves, independent oxidation reduction of ascorbic acid (Asc) and iron (Fe). Fe then binds to prolyl hydroxylase (PHD2) forming a complex with 2-oxoglutarate (2OG) and oxygen, this complex in turn hydroxylate HIFA with 2 by products succinate and carbon dioxide. Unbound hydroxylated HIFA binds with the von Hippel-Lindau-Elongin B-Elongin C complex ubiquinating HIFA for proteosomal degradation.

B. While in Hypoxia HIFA enters the nucleus for hydroxylation and escape degradation, PHDs binds to Fe, 2-oxoglutarate (2OG) and ascorbic acid but not oxygen. The protein inhibitor of growth 4 (ING4) binding to PHD2 may regulate HIFA transcriptional activity and blocks HIFA-HIFB complex binding. When HIFA and HIFB binding occurs the HIF dimer can activate the gene in the hypoxia response element (HRE) site, activating HIFA dependent genes where protein levels were upregulated like HIFA and PHD2.

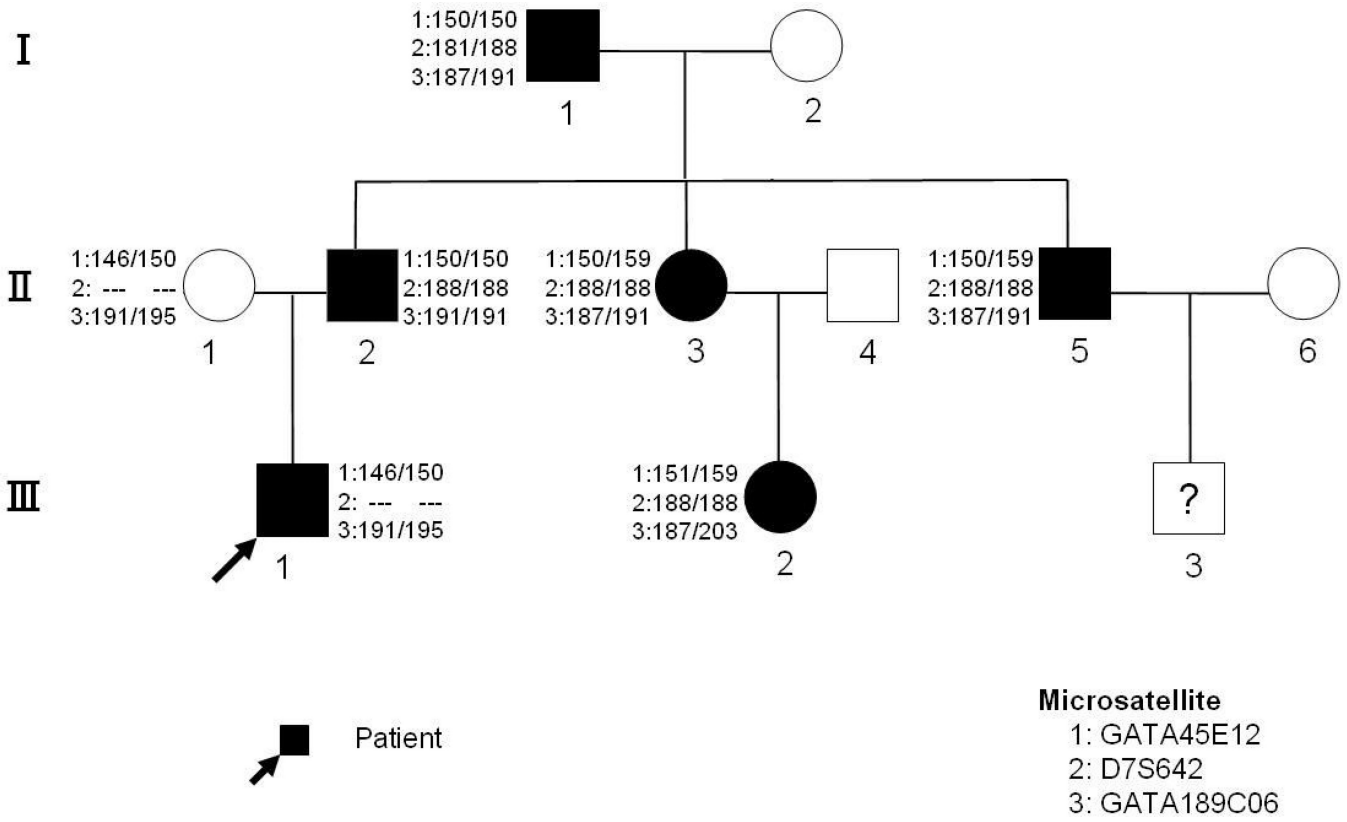

Figure 2. Pedigree chart of HAPE affected family

Shaded and unshaded figures indicate affected and unaffected individual respectively. Numeric paired values represent the genotype alignment of the 3 *eNOS* microsatellite markers used to rule out association with HAPE.