Hereditary Nonspherocytic Hemolytic Anemia caused by Red Cell Glucose-6-Phosphate Isomerase (GPI) deficiency in two Portuguese patients: clinical features and molecular study

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Short title: GPI deficiency in two Portuguese patients: clinical features and molecular study

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ABSTRACT

Glucose-6-phosphate isomerase (GPI) deficiency cause hereditary nonspherocytic hemolytic anemia (HNSHA) of variable severity in individuals homozygous or compound heterozygous for mutations in *GPI* gene. This work presents clinical features and genotypic results of two patients of Portuguese origin with GPI deficiency. The patients suffer from a mild hemolytic anemia (Hb levels ranging from 10 to 12.7 g/mL) associated with macrocitosis, reticulocytosis, hyperbilirubinemia, hyperferritinemia and slight splenomegaly. Genomic DNA sequencing revealed in one patient homozygosity for a new missense mutation in exon 3, c.260G>C (p.Gly87Ala), and in the second patient compound heterozygosity for the same missense mutation (p.Gly87Ala), along with a frameshift mutation resulting from a single nucleotide deletion in exon 14, c.1238delA (p.Gln413Arg fs*24). Mutation p.Gln413Arg fs*24 is the first frameshift null mutation to be described in GPI deficiency. Molecular modeling suggests that the structural change induced by the p.Gly87Ala pathogenic variant has direct impact in the structural arrangement of the region close to the active site of the enzyme.

Keywords:
Glucose-6-phosphate isomerase (GPI); GPI deficiency; Hemolytic anemia; Glycolysis; Portugal

Introduction

Glucose-6-phosphate isomerase (GPI, EC 5.3.1.9) is a homodimeric enzyme that catalyses the interconversion of glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P), in the second reaction step of the Embden–Meyerhof glycolytic pathway [1].

The enzyme is present ubiquitously in most organisms and expressed in all tissues, which has several physiological consequences in addition to its essential role in carbohydrate metabolism. GPI belongs to the moonlighting family of proteins having multiple functions/activities [2]. Molecular cloning and sequencing revealed that GPI share the same sequence to the protein known as neuroleukin (NLK), a neurotrophic factor that mediates differentiation and survival of embryonic spinal and sensory neurons [3,4]. GPI has been shown to work as autocrine motility factor (AMF), a tumor-secreted cytokine which stimulates cell migration and metastasis in an autocrine manner in various tumor cells [5-7]. GPI also shares sequence homology with the differentiation and maturation inducer for human myeloid leukemia HL-60 cells to terminal monocytic cells which indicates that GPI and maturation factor (MF) also share a common biological function, regulating differentiation and proliferation of human myeloid leukemic cells [2,8]. It was also found that chronic arthritis spontaneously developed by the K/BxN T cell receptor-transgenic mouse, with many features of human rheumatoid arthritis disease, is initiated by T cell recognition of GPI enzyme [9]. Moreover, immunization with human recombinant GPI protein induced arthritis in several mice models [10-12]. Recent literature also suggests a positive correlation between anti-GPI autoantibody and the arthritis disease in humans [13].

GPI deficiency (OMIM: 172400) is the second most frequent erythroenzymopathy in glycolysis and since the first report of the disease [14] about fifty cases have been reported throughout the world [1, 15]. Deficiency of the enzymatic activity occurs in individuals homozygous or compound heterozygous for GPI gene mutations and affects mostly erythrocytes causing hereditary nonspherocytic hemolytic anemia (HNSHA). Diagnosis is based on determination of the GPI activity in the red blood cells by enzyme quantitative assay. The major clinical features include variable degrees of jaundice, slight-to-moderate splenomegaly, an increased incidence of gallstones, and mild to severe anemia that is normochromic in most of the cases [1]. In severe cases GPI deficiency was associated with *hydrops fetalis* and neonatal death [16]. Few patients present with neuromuscular dysfunctions defined by muscle weakness and mental retardation [17].

The gene encoding GPI is located on chromosome 19q13.1 [18], contains 18 exons [19], and the cDNA of 1.9 kb translates a protein of 558 amino acids. The molecular characterization of GPI deficient variants shows that the gene defects are mostly missense mutations leading to protein instability or negatively influence the enzyme catalytic activity [1, 15]. Until now 34 GPI pathogenic variants have been documented [1, 20, 21] (HGMD, http://www.biobase-international.com), including 28 missense, three nonsense, two splicing and one recently described frameshift mutation (submitted).

A Portuguese GPI deficient patient was previously reported with hemolytic anemia associated with hyperbilirubinemia and splenomegaly, showing severe neurological impairment [22], however, at that time, molecular analysis could not be performed. In this work we present the clinical features and genotypic analysis of two additional unrelated Portuguese patients with GPI deficiency.

Methods

Patients

Patient 1 is a 31-year-old female diagnosed at the age of 10 with GPI deficiency. This woman has a palpable spleen (2 cm bellow the costal margin), a mild chronic hemolytic anemia (Hb 10-11.5 g/dL), macrocytosis (MCV 105 fL), reticulocytosis (180-200x10^9/L reticulocytes), unconjugated hyperbilirubinemia (total bilirubin 34 μmol/L; indirect 32 μmol/L) and iron overload (ferritin 450 ng/mL, transferrin saturation 66%) with no evidence of cardiac or hepatic hemosiderosis. Red blood cell GPI activity was 8 IU/g Hb (control 38 IU/g Hb). PK activity was 7.1 IU/g Hb (control 6.0 IU/g Hb) and G6PD activity was 6.8 IU/g Hb (control 6.2 IU/g Hb). She presents infrequent more severe hemolytic episodes associated with intercurrent infections.

Patient 2 is a 54-year-old male, presenting a mild hemolytic anemia (Hb 12.2 g/dL; reticulocytes 156x10^9/L), macrocytosis (MCV 100 fL), hyperferritinemia (650 ng/mL) and unconjugated hyperbilirubinemia (total bilirubin 62 μmol/L; indirect 59.1 μmol/L). GPI activity was 5 IU/g Hb (control 49 IU/g Hb). PK activity was 10 IU/g Hb (control 5.3 IU/g Hb) and G6PD activity was 11.2 IU/g Hb (control 9.4 IU/g Hb). His medical history included a total splenectomy after splenic injury at age of 18-year-old.

Hematological and biochemical analysis

Routine hematological studies were conducted with standard methods [23]. Red cell enzyme activities, expressed as IU/g Hb, were measured in hemolysates according to the methods recommended by the International Committee for Standardization in Haematology [24].

Genetic analysis

Genomic DNA was extracted from peripheral blood leukocytes of both patients and their relatives using standard methods. Polymerase chain reaction (PCR) to amplify GPI exons and adjacent intronic regions was performed with primers and conditions reported in Beutler et al. [25]. PCR products were purified with a ExoSap IT (Valencia, CA, USA) following the manufacturer's instructions and sequenced using the ABI Prism BigDye®Terminator V 1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the ABI 3130 genetic analyzer (Applied Biosystems). Screening of 50 unrelated normal individuals for the new missense mutation was performed by RFLP with the restriction endonuclease DdeI.

Structural Modelling

To better understand the structural implications of a novel missense mutation identified in the human GPI protein, we have used Modeller 9v15 [26] to build a three-dimensional model of the mutant protein. The model was generated based on the crystallographic structure of the human GPI with pdb code: 1NUH [27]. During the homology modelling process, Modeller starts by creating a single point mutation in the original structure and ends by optimizing and refining the mutant side-chain by conjugated gradient and simple Molecular Dynamics simulation [28]. In the end, PROCHECK [29] was used to perform a full stereochemical validation of the generated model.

Computational (in silico) predictive programs

The probable phenotypic effect of amino acid change for the new missense mutation was evaluated by using *in silico* tools commonly used for missense variant interpretation, including: PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/) [30], MutationAssessor [31] (http://mutationassessor.org), PROVEAN (http://provean.jcvi.org/index.php) [32], SIFT (http://sift.jcvi.org) [33], MutationTaster (http://www.mutationtaster.org) [34] and MutPred v.1.2 (http://mutpred.mutdb.org) [35], using the coordinates ENST00000356487, ENSP00000348877 and UniProtKB - P06744.

Results

The hematological, biochemical and molecular findings of the two unrelated Portuguese patients with GPI deficiency are summarized in Table 1. The two patients suffer from a mild hemolytic anemia (with Hb levels ranging from 10 to 12.7 g/mL) associated with macrocytosis, reticulocytosis, slight splenomegaly, hyperbilirubinemia and hyperferritinemia. The diagnosis of GPI deficiency was made after exclusion of the most common causes of hemolytic anemia and by the demonstration of a reduced GPI enzyme activity (about 80% in patient 1 and 90% in patient 2).

Sequencing of genomic DNA of patient 1 revealed homozygosity for a new missense mutation in exon 3, transversion c.260G>C, that changes codon GGT to GCT, leading to the amino acid substitution p.Gly87Ala. Sequence analysis showed both mother and father heterozygous for the variant c.260G>C. Patient 2 genomic DNA sequencing revealed compound heterozygosity for two different mutations: the same missense mutation c.260G>C (p.Gly87Ala), along with a single nucleotide deletion in exon 14, c.1238delA, resulting in the incorporation of incorrect amino acids into the protein after residue 413 and predicting premature termination of translation at codon 436 (p.Gln413Arg fs*24).

The novel missense mutation was confirmed by RFLP since c.260G>C change creates a restriction site for DdeI. Sequencing of the remaining GPI exons and respective adjacent regions showed no further nucleotide alterations in both patients, and the screening by RFLP of 50 unrelated normal individuals showed no mutated c.260C alleles. Moreover, the new variant was not found in ExAC, 1000Genomes or HGMD.

Structure analysis of the human GPI protein evidenced that Glycine 87 is placed at the end of α-helice 7 (composed by residues 76-86), preceding a loop region where Glutamate 93 and Arginine 95 are found (see Figure 1). Both these two residues are in close proximity with the active site of the GPI protein and heavily contributing to the stabilizing hydrogen bond network found in this region of the protein. Glycine residues, due to their stereochemical properties, are commonly found at the end and beginning of helical regions in proteins. Therefore, an Alanine mutant of a Glycine residue in such

regions is expected to perturb neighbor regions of the protein. In GPI, due to the proximity of Glycine 87 to the binding and active site of the GPI protein, one can propose that when mutated, the activity of the enzyme is affected. In fact, these observations corroborate the experimental data previously described for patient 1 where it is clear that a reduction in GPI activity is observed when this mutation is present. Moreover, the bioinformatic analysis using *in silico* tools to predict whether the new missense change p.Gly87Ala is damaging to the resultant protein function showed the following outputs (score / prediction): PolyPhen-2 0.856 / possibly damaging; MutationAssessor 3.595 / high impact; PROVEAN -4.55 / deleterious; SIFT 0.035 / damaging; MutationTaster 0.999 / disease causing; MutPred: probability of being deleterious 0.938.

Discussion

In two unrelated patients with chronic hemolytic anemia associated to GPI deficiency, two different mutations were identified: one novel missense mutation, c.260G>C (p.Gly87Ala), and one frameshift mutation due to a single nucleotide deletion, c.1238delA (p.Gln413Arg fs*24). Patient 1 is homozygous for mutation p.Gly87Ala, and the second patient is compound heterozygous for this same missense mutation (p.Gly87Ala) along with the frameshift variant c.1238delA (p.Gln413Arg fs*24).

The fact that mutation p.Gly87Ala was found in two unrelated patients, at the homozygous state in a non-consanguineous patient (which means, three mutated alleles in four unrelated chromosomes), would suggest the likelihood of a higher prevalence of this mutated allele among the Portuguese population. However, the study of 50 subjects from the general Portuguese population revealing no abnormal c.260C alleles did not enable to reach that conclusion.

Despite the severe reduction in GPI activity in red cells (about 80% and 90%), the nature of the anemia in both patients is mild (hemoglobin 10–12 g/dL). This finding is not in disagreement with previous reports, since genotype-phenotype correlations are difficult to establish in GPI deficiency. It is known that, in some patients, the degree of the GPI deficiency is not directed correlated with the severity of the anemia: patients with about half GPI activity display severe anemia while others with about 10% residual activity present with a mild to moderate anemia [1]. Moreover, patients with the same genotype and similar GPI activity reduction may have anemia of different severity [1].

The structural analysis of the mutant model of the GPI protein suggests that the p.Gly87Ala mutation has direct impact in the structural arrangement of the region close to the active site. Mutation p.Gly87Ala seems, not only to affect the stability of the α-helice 7, but more important, the stability of the hydrogen bond network found in the binding site of the GPI protein. This destabilization will therefore affect GPI activity as observed in the results presented in this work. Moreover, the use of multiple in silico algorithms for sequence variant interpretation predicts that the new missense change is damaging to the resultant protein function.

The frameshift mutation p.Gln413Arg fs*24 disrupts the open reading frame of the *GPI* mRNA transcript, predicting the formation of a truncated polypeptide 435 amino acids long, lacking about 25% of the COOH-terminal amino acid sequence. This abnormal polypeptide should not be compatible with dimerization, and thus, patient 2 can be considered functionally hemizygous for the missense mutation (p.Gly87Ala) present in the other chromosome. This could explain the similar reduction on GPI activity (about 85%) in both patients. This frameshift mutation was recently described in compound heterozygous state along with c.242G>A (p.Arg81Gln) in a GPI deficient patient from Spain with congenital hemolytic anemia (submitted).

Until now, only about 50 clinical cases of GPI deficiency-causing HNSHA have been reported in a variety of ethnic groups and populations among the world. Thirty five cases have been described to date at molecular level enabling the identification of 35 different GPI pathogenic variants (Table 2). Most (n=29) of these mutations are amino acid substitutions, not restricted to specific *GPI* gene regions. Exon 12 is the most affected gene region with five different mutations, followed by exon 18 with four mutations. Moreover, no specific common frequent mutations were found associated with GPI deficiency. The most common mutations are c.1028A>G (p.Gln343Arg) and c.1039C>T (p.Arg347Cys) reported in six chromosomes each (Table 2), representing 8.6% frequency (6 in 70) of abnormal alleles. Mutation c.1039C>T was found in individuals with a more diverse origin (Ashkenazi, German, Russian, African, Hispanic), suggesting recurrent *de novo* mutations at this C nucleotide, which is in accordance with its presence in a CpG dinucleotide. Moreover, from the 68 CpGs at the *GPI* cDNA (Ensembl Transcript ID ENST00000356487), 59 (86.8%) are affected by at least a nucleotide variation. Mutation c.1028A>G was found at the homozygous state in two subjects from different origins (Japan and Italy) and at compound heterozygous state in two German individuals, which seems to point for its higher prevalence in some populations. Five null mutations have

been found in GPI deficient patients to date (two splice site and three nonsense mutations), always in compound heterozygous state along with missense mutations [1]. Mutation c.1238delA (p.Gln413Arg fs*24) is the first frameshift null mutation to be described in GPI deficiency. The heterozygous state condition for all these null mutations reflects GPI as an essential housekeeping enzyme.

Neurological symptoms were not found in these two Portuguese patients, which indicate that the neurothrophic activity of the GPI enzyme was not affected by the aminoacid substitution p.Gly87Ala. Previously, a Portuguese patient was reported to show severe neuromuscular impairment [22]. However, this GPI variant was not characterized at the molecular level and a sample is currently unavailable. It remains unexplained why some GPI mutations cause neurological symptomatology and others do not. From all patients reported with enzymatic deficiency in GPI, neurologic impairment was observed in at least five [1]. Until now, only two patients (GPI variants Homburg and Mount Scopus) with neurological symptoms have been characterized at the molecular level [1]. Patient GPI Homburg is compound heterozygous for two missense mutations, p.His20Pro and p.Leu339Pro, that are likely to affect the folding and activity of the enzyme [17, 36]. Based on this case, where the neuromuscular symptoms were directly related to the enzyme deficiency, the authors proposed GPI mutations that lead to incorrect folding destroys both catalytic and neurotrophic (NLK) activities of GPI protein; those alterations at the active site, or at or close to the subunit interface that destabilize the dimer but not necessarily the monomer, allow a correct folding retaining the neurotrophic properties of the molecule [17]. In concordance with this hypothesis, molecular modelling suggest that the GPI missense mutation p.Gly87Ala identified in the two Portuguese patients exhibiting no neurological symptoms has direct impact in the region close to the active site. Similarly, previous reported patients carrying mutations at or close to the active site, including H389R [17], R273H [37], and S278L [37] did not show neurological impairment.

In conclusion, this report describes the clinical features and the molecular etiology of two GPI deficient Portuguese patients. We found one patient homozygous for the novel GPI missense mutation c.260G>C (p.Gly87Ala) and a second patient compound heterozygous for p.Gly87Ala and the frameshift p.Gln413Arg fs*24. Molecular modeling suggests that the structural changes induced by the p.Gly87Ala mutation affects the active site of the enzyme. Both patients showed no neurological impairment.

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Legends

Figure 1 – Cartoon representation of the human GPI protein (1NUH) (A). Zoom of the binding site region of the human GPI protein (1NUH) (B) and of the mutant homology model generated using Modeller (C). The side-chains of the residues belonging to the catalytic site of the GPI protein are represented as green sticks, while the side-chains of the residues belonging to the binding region of the catalytic site of the GPI protein are represented as yellow sticks. Glycine 87 and its mutant counterpart Alanine 87 are also represented respectively in figures (B) and (C) on the right side as purple sticks. All images were prepared using the software PYMOL [44].

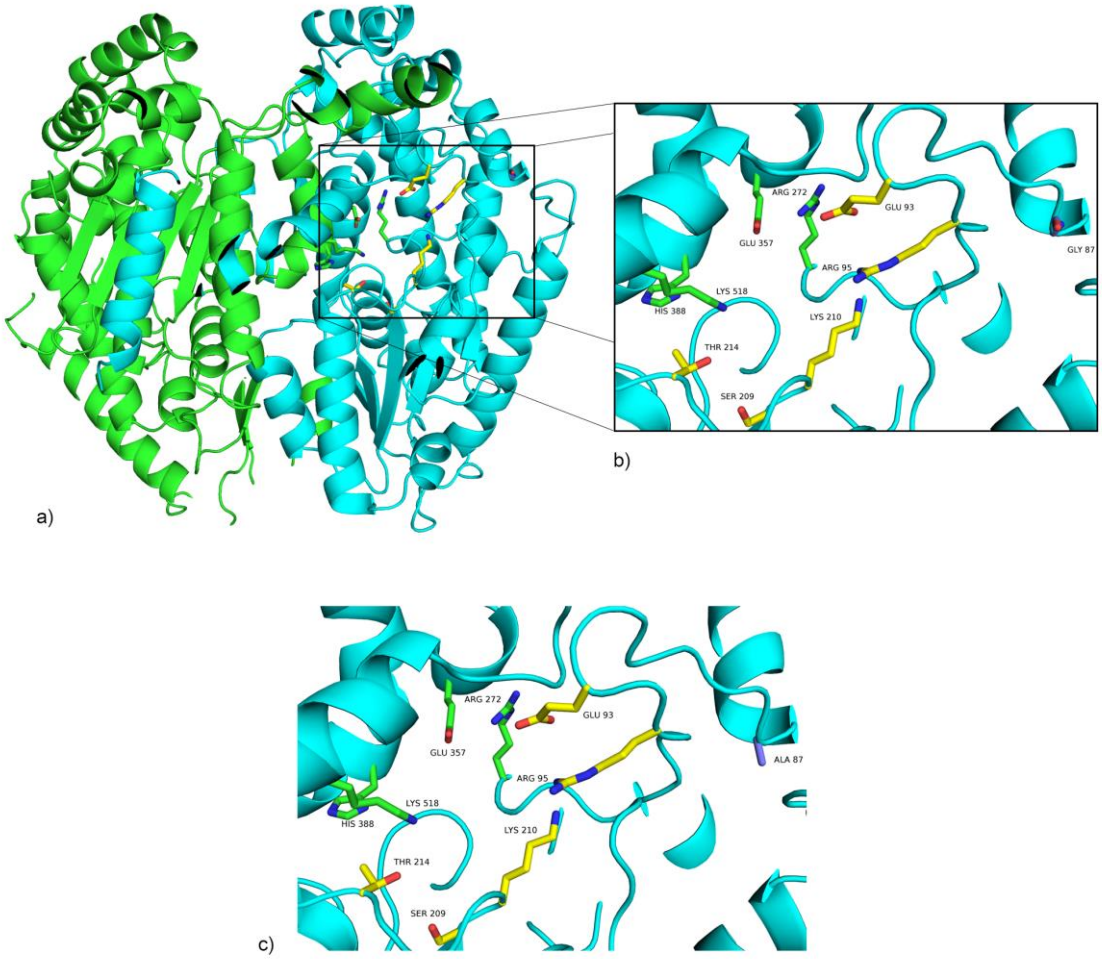

Table 1: Hematological and biochemical data in the two Portuguese patients with GPI deficiency.

Parameters	**Patient 1**	**Patient 2**	**Reference values**
Age	31 years	54 years	-
Sex	Female (F)	Male (M)	-
RBC ($x10^{12}$/L)	2.88	3.67	M: 4.5-5.5 F: 3.8-4.8
Hb (g/dL)	10-11.5	12.2	M:13-17.5 F: 12-16
MCV (fL)	105	102	80-100
MCH (pg)	34.7	33.3	27-32
MCHC (g/dL)	32.8	32.7	32-35
RDW (CV %)	11	10.8	11.6-14
WBC ($x10^9$/L)	3.6	14.5	4-10
Platelets ($x10^9$/L)	313	530	150-400
Reticulocytes ($x10^9$/L)	183 (5.7%)	156 (4%)	50-100
Serum Ferritin (ng/mL)	450	650	9-120
Unconjugated Bilirubin (μmol/L)	32	59.1	<12.8
G6PD (IU/g Hb)	6.8	11.2	6.5-13
PK (IU/g Hb)	9.1	10	9-14
GPI (IU/g Hb)	8	5	45-75

Table 2. Mutations reported until now in patients with GPI deficiency.

#	Exon	Mutation	Consequence	Origin	Zygosity	N chr	Ref
1	1	c.14C>G	p.Thr5Ile	Japanese	Homozygous	2	38
2	1	c.43C>T	p.Gln15Stop	Germany	Compound ht	1	1
3	1	c.59A>C	p.His20Pro	Germany	Compound ht	1	17
4	3	c.223A>G	p.Arg75Gly	White Am	Compound ht	1	25
5	3	c.242G>A	p.Arg81Gln	Spanish	Compound ht	1	submitted
6	3	c.247C>T	p.Arg83Trp	Indian Am	Homozygous	2	37
7	3	c.260G>C	p.Gly87Ala	Portuguese	Homozygous Compound ht	3	this study
8	4	c.286C>T	p.Arg96Stop	Italian Russian White Am	Compound ht Compound ht Compound ht	3	39 25 25
9	4	c.301G>A	p. Val101Met	Italian	Homozygous	2	39
10	4	c.341A>T	p.Asp114Val	Spanish	Compound ht	1	21
11	5	c.475G>A	p.Gly159Ser	?English	Compound ht	1	40
12	6	c.584C>T	p.Thr195Ile	Italian Italian	Compound ht Compound ht	2	39
13	7	c.663T>G	p.Asn221Lys	Spanish	Compound ht	1	21
14	7	c.671C>T	p.Thr224Met	Japanese Black Am	Homozygous Compound ht	3	38 37
15	10	c.818G>A	p.Arg273His	Black Am	Compound ht	1	37
16	10	c.833C>T	p.Ser278Leu	White Am	Compound ht	1	37
17	11	c.898G>C	p.Ala300Pro	Hispanic	Compound ht	1	25
18	12	c.970G>A	p.Gly324Ser	Turkish	Homozygous	2	1
19	12	c.1016T>C	p.Leu339Pro	German	Compound ht	1	17
20	12	c.1028A>G	p.Gln343Arg	Italian Japanese German German	Homozygous Homozygous Compound ht Compound ht	6	39 38 41 1
21	12	c.1039C>T	p.Arg347Cys	Ashkenazi German Russian Black Am Hispanic	Homozygous Compound ht Compound ht Compound ht Compound ht	6	25 41 25 37 25
22	12	c.1040G>A	p.Arg347His	?English Spanish	Compound ht Homozygous	3	40 42
23	13	c.1124C>G	p.Thr375Arg	Japanese	Compound ht	1	38

24	13	c.1166A>G	p.His389Arg	German	Compound ht	1	17
25	14	c.1238delA	p.Gln413Arg fs*24	Portuguese Spanish	Compound ht Compound ht	2	this study submitted
26	Ivs15	Ivs15(-2)A>C	Splice site	German	Compound ht	1	41
27	16	c.1415G>A	p.Arg472His	Hispanic Turkish	Homozygous Homozygous	4	25 1
28	16	del1473-ivs16(+2)	Splice site	Italian	Compound ht	1	39
29	16	c.1459C>T	p.Leu487Phe	White Am Indian	Compound ht Homozygous	3	37 20
30	17	c.1483G>A	p.Glu495Lys	Black Am	Compound ht	1	37
31	17	c.1538G>A	p.Trp513Stop	German	Compound ht	1	41
32	18	c.1549C>G	p.Leu517Val	German	Compound ht	1	17
33	18	c.1574T>C	p.Ile525Thr	?English	Homozygous	2	40
34	18	c.1615G>A	p.Asp539Asn	Japanese Japanese Dutch	Homozygous Compound ht Homozygous	5	38 38 43
35	18	c.1648A>G	p.Lys550Glu	Spanish Spanish	Homozygous Homozygous	4	submitted 21

Abbreviations: ht, heterozygous; Am, American; N chr, Number of chromosomes.