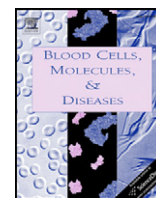


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## Chronic hemolytic anemia is associated with a new glucose-6-phosphate dehydrogenase in-frame deletion in an older woman

Licínio Manco<sup>a,b</sup>, Janet Pereira<sup>a</sup>, Luís Relvas<sup>a</sup>, Umbelina Rebelo<sup>a</sup>, Ana Isabel Crisóstomo<sup>c</sup>, Celeste Bento<sup>a</sup>, M. Letícia Ribeiro<sup>a,\*</sup>

<sup>a</sup> Unidade de Hematologia Molecular, Centro Hospitalar de Coimbra, Coimbra, Portugal

<sup>b</sup> CIAS / Departamento de Ciências da Vida, Universidade de Coimbra, Coimbra, Portugal

<sup>c</sup> Serviço de Hematologia, Hospitais da Universidade de Coimbra, Coimbra, Portugal

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### ABSTRACT

Glucose-6-phosphate dehydrogenase (G6PD) deficiency, an X-linked disorder, is usually observed in hemizygote males and very rarely in females. The G6PD class 1 variants, very uncommon, are associated with chronic hemolytic anemia. Here we report a Portuguese woman who suffered in her sixties from a chronic hemolytic anemia due to G6PD deficiency. Molecular studies revealed heterozygosity for an in-frame 18-bp deletion, mapping to exon 10 leading to a deletion of 6 residues, 362–367 (LNERKA), which is a novel G6PD class 1 variant, G6PD Tondela. Two of her three daughters, asymptomatic, with G6PD activity within the normal range, are heterozygous for the same deletion. The patient's leukocyte and reticulocyte mRNA studies revealed an almost exclusive expression of the mutant allele, explaining the chronic hemolytic anemia. Patient whole blood genomic DNA HUMARA assay showed a balanced pattern of X chromosome inactivation (XCI), but granulocyte DNA showed extensive skewing, harboring the mutated allele, implying that in whole blood, lymphocyte DNA, with a very long lifetime, may cover up the current high XCI skewing. This observation indicates that HUMARA assay in women should be assessed in granulocytes and not in total leukocytes.

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### Introduction

X chromosome inactivation (XCI) is an epigenetic process, unique in mammals, by which one of the two X chromosomes in each cell is inactivated in females early in embryogenesis [1]. Therefore, women are a mosaic of paternal and maternal active X chromosome and a theoretical 1:1 ratio of two cell lines with inactive maternal and paternal X chromosome could be expected. The XCI ratio is usually assessed analyzing protein variants directly in heterozygous females' cells [2], transcribed mRNA expression [3] or DNA methylation status of polymorphic X-linked genes, such as the human androgen receptor (HUMARA) gene [4]. Deviation from the theoretical 1:1 ratio between the 2 parental alleles is called skewing. Several reports using the HUMARA assay show that the incidence of skewing is relatively low at birth and in adult non-hematopoietic tissues, but higher and age-dependent in hematopoietic cells, with 30–40% of healthy elderly women reported to have skewing (greater than 75% expression of one parental X chromosome) [5–12]. T lymphocytes show less evidence of skewing with age than neutrophils, presumably reflecting the neutrophils' short half-life, whereas T lymphocytes are long-living

cells, and in consequence, some T cells are produced near the time of study but others are derived from earlier stem cells [11–13]. This age-related skewing in blood cells has been attributed to clonality as a consequence of hematopoietic stem cell senescence [5,7,9].

Swierczek et al. report a discrepancy between the skewed XCI observed in blood cells of 45 elderly women (ages 65–92 years; mean, 81.3 years) using the methylation-based HUMARA assay and a transcriptional based assay: with HUMARA assay in granulocyte DNA they found an age-dependent skewing at this locus, and preferential methylation of one allele; using a quantitative polymerase chain reaction (qPCR) transcriptional clonality assay to measure allele-specific expression of 5 genes, subject to X-inactivation, they did not find clonal or oligoclonal hematopoiesis in 37 informative healthy older women compared with younger women. The authors concluded that skewed allelic methylation ratios in elderly women observed by HUMARA assay are the result of an age-related deregulated methylation of the HUMARA gene locus, but in the absence of co-morbid conditions, healthy individuals do not exhibit clonality or oligoclonality of hematopoiesis with aging [14]. Busque et al. studied 100 women from elderly and young cohorts combined (mean age, 64 years) using HUMARA and 2 independent transcription clonality assays (one based on a quantitative allele-specific PCR and the other based on TaqMan-SNP) and found similar skewing incidences with the 3 different methods ( $40\% \pm 2\%$ ). The authors

\* Corresponding author at: Departamento de Hematologia, Centro Hospitalar de Coimbra, EPE, 3041-853 Coimbra, Portugal. Fax: +351 239 717216.

E-mail address: [leticia.ribeiro@chc.min-saude.pt](mailto:leticia.ribeiro@chc.min-saude.pt) (M.L. Ribeiro).

conclude that their results validate previous studies in which HUMARA assay document age-dependent skewing in females and that skewing of XCI ratio seen in blood cells of aging women is a genuine biologic phenomenon [6].

Until recently it has been assumed that the XCI skewing intensity would be the same in all tissues. However, data from Bolduc et al. shows that the skewing extent varies by tissue type, although maintaining the direction (toward maternal or paternal allele) across tissues, in accordance with the concept that the primary XCI occurs during early development, prior to the splitting of the inner cell mass and to tissue specification [10].

In general, skewing may not have any biologic consequence; however, extremely skewed XCI in blood cells has been reported in severely affected females for a X-linked trait. Several cases of symptomatic carrier females with specific X-linked gene mutations, in whom the X chromosome carrying the normal allele is predominantly inactivated, have been described in hemophilia A, [15] hemophilia B, [16] sideroblastic anemia [17,18] and glucose 6-phosphate dehydrogenase (G6PD) deficiency [19]. Moreover, studies in Chinese women heterozygous for G6PD variants showed that the frequency of G6PD deficiency increased with age, and that G6PD activity was related to the degree of skewing of XCI determined by the HUMARA assay [20].

G6PD catalyzes the first step of the pentose phosphate pathway and is the product of a housekeeping X-linked gene ubiquitous in its expression in mammalian tissues [21]. More than 160 mutations have been identified associated with G6PD deficiency, the great majority consisting in single point mutations causing amino acids replacements [22]. Few small in-frame nucleotide deletions have been described, all in males, associated with chronic nonspherocytic hemolytic anemia (CNSHA): 5 deletions of 1 amino acid, 1 deletion of 2 amino acids and 1 deletion of 8 amino acids [22]. The remark that nonsense, frameshifts or splicing mutations had not been found in association with G6PD deficiency is probably due to the fact that the hemizygous state for such null mutations, responsible for total absence of G6PD activity, is incompatible with life. The exception is the deletion of the invariant dinucleotide “ag” at the intron 10 acceptor splice-site, reported by Xu et al. which presumably causes a deletion of 3, 9 or 20 amino acids due to the activation of cryptic splicing sites in exon 11 [23].

In this work we illustrate the age-dependent skewing in a Portuguese woman heterozygous for a novel G6PD class 1 variant, who presented at the age of 74 years with chronic hemolytic anemia. Differences in clinical manifestations between the patient and her two daughters with the same genotype are explained by the G6PD mutated allele expression assessed by reverse transcription-polymerase chain reaction (RT-PCR) method.

## Materials and methods

### Subjects

A 74 year old Portuguese woman presented with a history of chronic hemolytic anemia with palpable spleen 5 cm below the left costal

margin, identified during the previous decade. The anemia was macrocytic with reticulocytosis: Hb = 9.4 g/dL; MCV = 114 fL; reticulocytes = 28%; unconjugated serum bilirubin 42 micromol/L (normal <17); serum ferritin 400 ng/mL; G6PD activity in erythrocytes = 16.6% of normal. The patient had no history of anemia during her three full-term pregnancies. The daughters (D1, D2, and D3), aged 37, 36 and 35 year old, have normal hematological parameters and were also investigated (Table 1).

### Hematological studies and enzyme activity analysis

Blood was obtained by venipuncture and granulocyte and lymphocyte fractions were isolated by Histopaque density gradient as recommended by the manufacturer (Sigma-Aldrich). Hematological parameters were determined by standard methods [24]. G6PD enzyme activity assays in the red blood cells and leukocytes lysates were performed by quantitative spectrophotometer analysis according to the recommendations of the International Committee for Standardization in Hematology (ICSH).

Electrophoresis of partial purified lysates was performed in horizontal 20% starch gels, pH 7.6, and the G6PD mobility was examined using a functional method (Harris and Hopkinson, 1976).

### Molecular studies

#### Genomic DNA

After informed consent, genomic DNA was extracted from EDTA peripheral blood (PB), and from granulocytes and lymphocytes fractions using standard molecular protocols. The G6PD coding exons 2–13 were amplified by polymerase chain reaction (PCR) and tested for the common G6PD deficient variants, as previously described [25–27]. The amplicons were subjected to single strand conformation polymorphism (SSCP) analysis and to direct sequencing by dideoxy chain termination reaction, using the ABI Prism 310 genetic analyser (Applied Biosystems).

#### XIST gene

The Xist gene, involved in X chromosome inactivation, was tested for the presence of the –43C→G mutation in the minimal promoter region, using the restriction endonuclease HhaI [28].

#### RNA studies

Total RNA was isolated from top half of packed red blood cells and leukocytes in PB by RNeasy plus mini Kit (Qiagen) as per manufacturer's recommendations. The isolated RNA was reverse transcribed, primed with random hexamers, using First-Strand cDNA synthesis kit (Amersham Biosciences) as described by the manufacturer. The cDNA was subjected to PCR using forward primer 5'-acttttgacgctgctc-3' and reverse primer 5'-agaagacgtccaggatgagg-3'. The resulting 331 bp amplicons, containing exons 9–11, were electrophoresed through horizontal 10.0% bis-acrylamide gel. The intensity of the reverse transcription-polymerase chain reaction (RT-PCR) fragments was measured and integrated using the genescan software “ImageMaster 1D Elite, version 2.01” (Hoefer Pharmacia

**Table 1**  
Hematological and biochemical analysis summarised.

Patient	Age (years)	Hb (g/dL) n.r. 12.0–16.0	MCV (fl) n.r. 80–96	Retics (%) n.r. <2.0%	Leuk ( $\times 10^9/L$ )	Neutr ( $\times 10^9/L$ )	Lymph ( $\times 10^9/L$ )	Plat ( $\times 10^9/L$ )	Total Bilirubin ( $\mu\text{mol/L}$ ) n.r. <22	G6PD RBC (% of N)	G6PD leukocytes (% of N)
Patient	74	9.4	114	28	9.81	4.69	1.82	254	26.8	16.6	<10%
Daughter1	37	12.3	96	3.7	8.15	4.80	2.30	269	14.3	85.5	100
Daughter2	36	11.9	94	2.8	6.6	4.49	1.74	285	10.4	115.5	n.d.
Daughter3	35	12.1	92	0.8	6.8	4.30	2.05	271	8.8	100	n.d.

Hb: hemoglobin (normal values: adult females, 120–160 g/L); MCV: mean cell volume (lowest normal values: adults, 80 fL); Retics: reticulocytes (normal values: 0–2%); n.d.: not determined; n.r.: normal; Leuk: leukocytes (normal values: 4–10  $\times 10^9/L$ ); Neutr: neutrophils (normal values: 2–7  $\times 10^9/L$ ); Lymph: lymphocytes (normal values: 1–3  $\times 10^9/L$ ); Plat: platelets (normal values: 150–400  $\times 10^9/L$ ).

Biotech). The RT-PCR products were also subjected to Sanger's direct sequencing using the ABI Prism 310 genetic analyzer (Applied Biosystems).

#### HUMARA methylation assay

Genomic DNA isolated from blood total leukocytes, granulocytes and lymphocytes fractions was subjected to the HUMARA methylation assay as previously reported [4]. Briefly, genomic DNA was treated overnight with and without the methylation-sensitive endonuclease *HhaI* and subsequently subjected to PCR using primers previously described flanking the short tandem repeat (STR) polymorphism in the HUMARA gene [12]. The PCR products were analyzed using the automatic sequencer AlExpress II (Amersham Pharmacia Biotech). The allele's size and area under the curve (AUC) was measured with the software ALFwin™ Fragment Analyser 1.00 (Amersham Pharmacia Biotech). The ratio of the active/inactive X chromosome was estimated according to the equation defined by Bolduc et al. [10]:

$$P_{\text{sup}} = 1 - \left( \frac{\frac{A/(A+a)}{A'/(A'+a')}}{\frac{A/(A+a)}{A'/(A'+a')} + \frac{a/A+a}{a'/A'+a'}} \right)$$

A and A' represent the AUC of the superior HUMARA allele from digested and undigested sample, respectively; a and a' represent the AUC of the inferior HUMARA allele for the digested and undigested sample, respectively.  $P_{\text{sup}}$  indicates the percentage of cells expressing the X chromosome bearing the superior allele. A  $P_{\text{sup}}$  score equal or greater than 75% or equal or less than 25% indicates skewing. The  $P_{\text{sup}}$  specifies the direction of skewing:  $P_{\text{sup}} > 50\%$  indicates the X chromosome bearing the superior allele is predominant, while  $P_{\text{sup}} < 50\%$  indicates the opposite [10].

## Results and discussion

We report a clinical case of a Portuguese woman referred to our out-patient clinic at the age of 74 years with macrocytic chronic hemolytic anemia due to G6PD deficiency. Her G6PD activity in red blood cells was about 17% of normal and it was severely reduced (<10% of the normal) in leukocytes. In spite of that, the patient has no clinical evidence of decreased leukocyte functions, namely increased susceptibility to bacterial or fungal infections, as it has been described in patients with granulocytes G6PD activity below 5% of normal [29]. Her three daughters have erythrocytes G6PD activity within the normal ranges (Table 1). The G6PD starch-gel electrophoresis revealed only the normal migrating G6PD B band (data not shown).

#### Genomic DNA studies

Patient and daughters' genomic DNA was tested for G6PD A variant (A376G) with *FokI*, confirming the presence of the G6PD B alleles. The amplicon containing exon 10 has a shift on SCCP gel electrophoresis, implying the presence of a putative mutation, and the direct sequencing was consistent with an 18-bp deletion at the heterozygous state (data not shown). The 10.0% acrylamide gel electrophoresis of the PCR fragment spanning exon 10 showed an abnormal fast moving band, confirming the patient to be heterozygous for the 18 bp deletion (Fig. 1A). Two of her daughters (D1 and D2) were also heterozygous for the 18 bp deletion; the third daughter (D3) was homozygous for the wild type allele (Fig. 1A). The sequence analysis of the promoter, whole coding exons and flanking introns regions in patient DNA excluded any additional mutations.

The -43C→G XIST promoter mutation, associated with nonrandom X chromosome inactivation [28], was not found in patient and her daughters.

#### mRNA studies

Patient reticulocytes and leukocytes G6PD mRNA expression analysis spanning G6PD exons 9–11 showed markedly increased expression of the mutant allele relative to the normal allele (>95% in reticulocytes and ~80% higher in leukocytes), while daughter D1 mRNA exhibited similar expression of both mutated and normal transcripts (~50% each in both leukocytes and reticulocytes) (Fig. 1B).

Probably due to different methods sensitivity, RT-PCR amplicon G6PD exon 10 sequence analysis on patient's reticulocytes and leukocytes failed to show the normal nucleotide sequence, reflecting the markedly increased expression of the abnormal mRNA in both hematopoietic cells. Moreover, the nucleotide sequencing profile confirmed the presence of the 18-bp in-frame deletion (Fig. 1C) that is predicted to remove 6 amino acids, p.362-p.367del (LNERKA). These 6 residues are located in the loop connecting  $\beta$ -strands J and K that form part of the  $\beta$ -sheet that constitutes a scaffold for binding of the structural NADP<sup>+</sup>, and extends across G6PD dimer interface. Two perfect 8-bp repeats (GCAAGGCC) mark the endpoints of the pathogenic deletion, the 5' breakpoint between nucleotides c.1076 and c.1083 and the 3' breakpoint between nucleotides c.1094 and c.1101 (Fig. 1C).

Therefore, mRNA studies support skewed XCI in the patient's erythrocytes and leukocytes and a balanced XCI in daughter D1 leukocytes and erythrocytes, which is in accordance with their G6PD activity and clinical phenotypes.

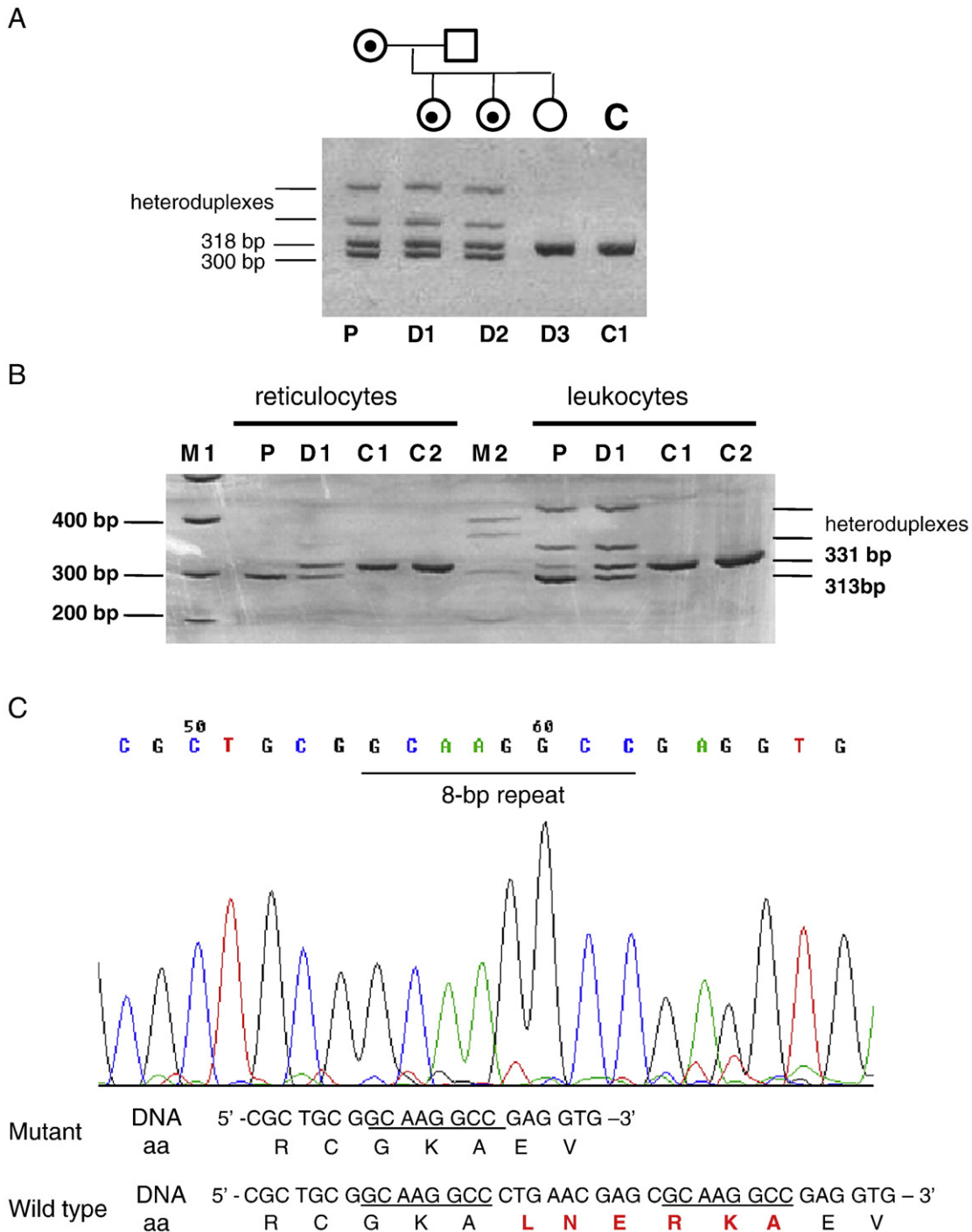
#### HUMARA assays

The pattern of X chromosome inactivation in patient genomic DNA from whole blood (leukocytes), assessed by the methylation-based HUMARA clonality assay (Fig. 2), showed a similar PCR amplification amount of two HUMARA alleles (278- and 287-bp) in accordance to a balanced pattern of XCI (Fig. 2C-1), with a  $P_{\text{sup}}$  score of 40%, according to the equation reported by Bolduc et al. [10]. Thus, patient HUMARA data was not in keeping with the blood G6PD mRNA studies which point to a marked increase expression of the mutant allele, accounting for the late development of her chronic hemolytic anemia.

To elucidate the discordance between mRNA and HUMARA studies we performed lineage specific HUMARA analysis on DNA isolated from granulocytes and lymphocytes fractions. In the granulocytes we found a skewing of X-inactivation towards the 278-bp HUMARA allele (Fig. 2C-2):  $P_{\text{sup}}$  score of 16%, which means that the X chromosome bearing the inferior HUMARA allele, associated to the G6PD mutated allele, is predominantly expressed. In lymphocytes (Fig. 2C-2)  $P_{\text{sup}} = 54\%$  indicates no skewing, similar to the HUMARA DNA studies in the whole blood ( $P_{\text{sup}} = 40\%$ ), implying that the presence in whole blood of a significant number of T cells derived from earlier stem cells, before the acquired skewing occurred, masked the high XCI skewing in the granulocytes. These results are in accordance to previous reports showing that skewing was markedly increased in older women's granulocytes, but not in T cells [11–13]. As lymphocytes have a long circulatory lifetime, these data are consistent with XCI skewing developing with age, implying that HUMARA assay in whole blood is not reliable to quantify the XCI skewing in older women.

The higher mRNA expression of the G6PD mutated allele in patient leukocytes (~80%) is associated with the extreme X-skewed inactivation in the granulocytes (Table 1). The presence of the long-lived lymphocytes may explain the small normal band (~20% expression) observed in RT-PCR analysis from leukocytes, corresponding to the wild type G6PD allele, which is more intense than the faint normal band obtained from reticulocytes cDNA (Fig. 1B).

The HUMARA pattern in whole blood DNA from daughter D1 showed an almost balanced XCI pattern between the maternal and paternal allele:  $P_{\text{sup}} = 73\%$ , indicating no skewing but a more active father's 290-bp superior allele (Fig. 2C-1) in agreement with her



**Fig. 1.** (A) Electrophoretic analysis of genomic DNA PCR fragments from G6PD exon 10. Mother and daughters 1 and 2 showed the normal band and a faster moving band corresponding to the G6PD allele containing the 18-bp deletion. (B) RT-PCR products from patient and daughter 1 reticulocytes and leukocytes spanning G6PD exons 9–11 showing the normal band (331-bp) and the fast moving band (313-bp) corresponding to the abnormal allele preferential expressed in patient's peripheral blood. (C) Patient leukocytes cDNA sequence spanning the 18-bp deletion, within G6PD exon 10, showing the 8-bp repeat (GCAAGGCC) that marks the pathogenic deletion endpoints. M1, 1 kb marker (Invitrogen); M2, marker (pBR322 digested with *Hinf*I); P, patient; D1, daughter 1; D2, daughter 2; D3, daughter 3; C1 and C2, control samples.

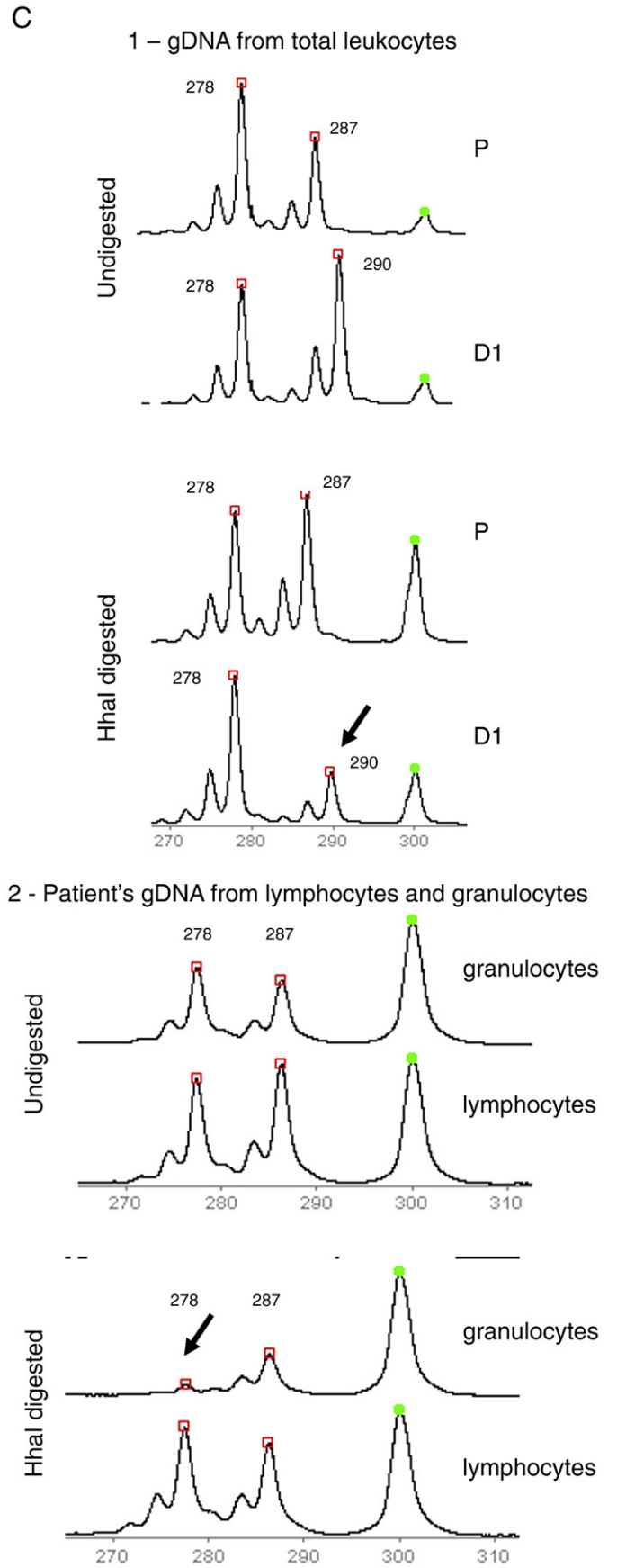
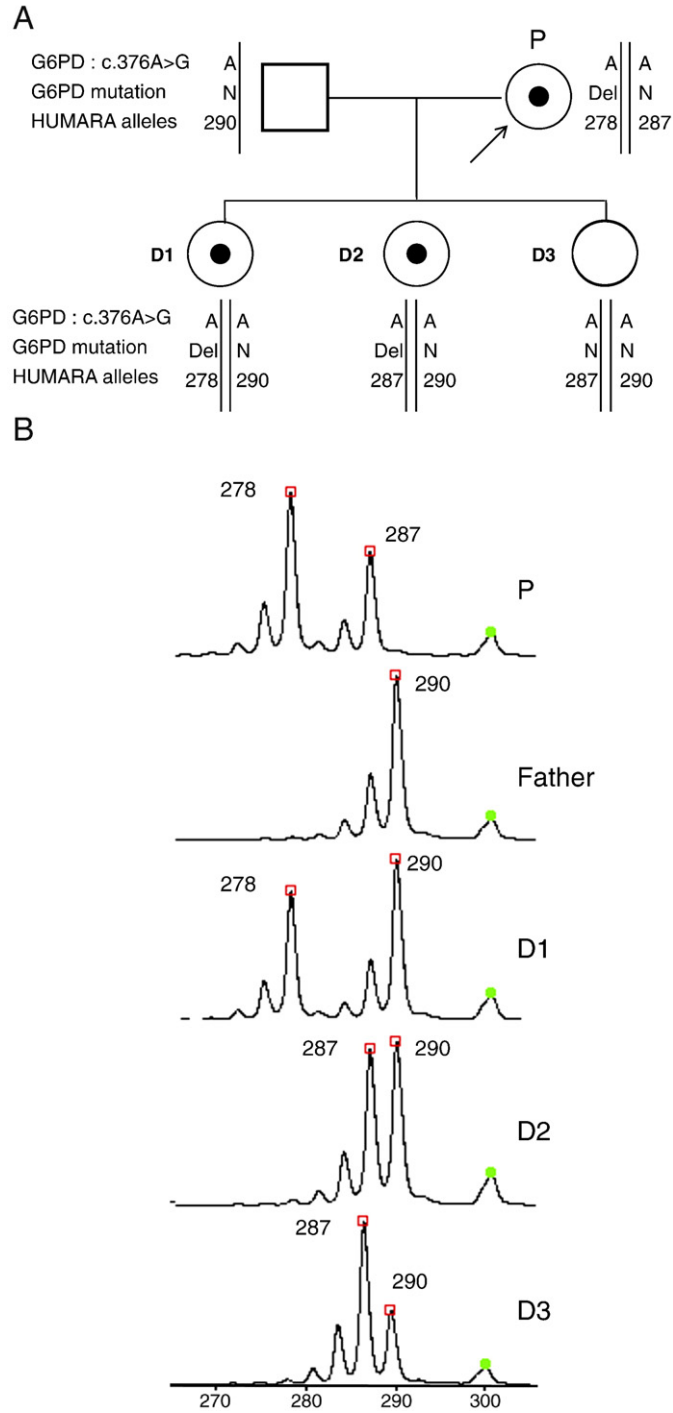
mRNA expression of both mutated and normal transcripts in leukocytes and reticulocytes and with her normal hematological parameters and G6PD activity.

Curiously, daughters D1 and D2, both heterozygous for the mutant G6PD allele, present different mother's HUMARA alleles: 278- and 287-bp, respectively (Fig. 2B), implying a recombination event between HUMARA and G6PD loci located far apart (about 86.8 Mb) in the X chromosome. As D3 is homozygous for the G6PD wild type allele and her X haplotype includes the 287-bp HUMARA allele, the

most probable explanation is that the 278-bp androgen receptor allele segregates with G6PD deficient-allele, which means that daughter D1 and D3 carry non-recombinant alleles and daughter D2 the recombinant allele (as shown in Fig. 2A).

Thus, in a Portuguese woman with a chronic hemolytic anemia exacerbation in her late sixties, we identified a new G6PD-deficient class I variant due to a 6 residues deletion near the enzyme dimer interface, in the context of the G6PD B haplotype (376A). We named the new class 1 variant G6PD Tondela, after patient place of birth.

The patient's chronic hemolytic anemia is caused by an almost exclusively expression of the mutant G6PD allele, as shown in reticulocytes and leukocytes G6PD mRNA studies. HUMARA assays



**Fig. 2.** (A) The Mendelian analysis of the HUMARA alleles showing alleles 278- and 287-bp in patient' DNA. The 3 daughters inherited the 290-bp allele from the father and, from the mother daughter 1 inherited the 278-bp allele and daughters 2 and 3 the 287-bp allele. (B) Patient's and relatives HUMARA alleles detected using the automatic sequencer ALExpress II (Amersham Pharmacia Biotech). (C) HUMARA methylation assay in patient and daughter 1 performed in genomic DNA from peripheral whole blood (1), lymphocytes and granulocytes (2). The arrow indicates the predominantly active chromosome: as in the inactive X chromosome the *HhaI* site is methylated, no digestion occurs and PCR amplification takes place; in the active X chromosome, the regular *HhaI* digestion leads to disruption of the HUMARA gene, preventing PCR amplification. P, patient; D1, daughter 1; D2, daughter 2; D3, daughter 3.

Fig. 2 (continued).

showed an extensively skewed towards the chromosome harboring the mutated allele in DNA from granulocytes, but not from lymphocytes. Taken together, these data suggest a skewed X chromosome inactivation process, which seems to aggravate with age, as the deviation from the normal ratio was only observed in granulocytes, with a short half-life.

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