Clinical Severity of PGK1 Deficiency Due To a Novel p.E120K Substitution Is Exacerbated by Co-inheritance of a Subclinical Translocation t(3;14)(q26.33;q12), Disrupting *NUBPL* Gene

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Abstract Carriers of cytogenetically similar, apparently balanced familial chromosome translocations not always exhibit the putative translocation-associated disease phenotype. Additional genetic defects, such as genomic imbalance at breakpoint regions or elsewhere in the genome, have been reported as the most plausible explanation.

By means of comprehensive molecular and functional analyses, additional to careful dissection of the t(3;14)(q26.33;q12) breakpoints, we unveil a novel X-linked PGK1 mutation and examine the contribution of these to the extremely severe clinical phenotype characterized by hemolytic anemia and neuromyopathy.

The 3q26.33 breakpoint is 40 kb from the 5' region of tetratricopeptide repeat domain 14 gene (*TTC14*), whereas the 14q12 breakpoint is within IVS6 of nucleotide-binding protein-like gene (*NUBPL*) that encodes a mitochondrial complex I assembly factor. Disruption of *NUBPL* in translocation carriers leads to a decrease in the corresponding mRNA accompanied by a decrease in protein level. Exclusion of pathogenic genomic imbalance and reassessment of familial clinical history indicate the existence of an additional causal genetic defect. Consequently, by WES a novel mutation, c.358G>A, p.E120K, in the X-linked phosphoglycerate kinase 1 (*PGK1*) was identified that segregates with the phenotype. Specific activity, kinetic properties, and thermal stability of this enzyme variant were severely affected. The novel *PGK1* mutation is the primary genetic alteration underlying the reported phenotype as the translocation per se only results in a subclinical phenotype. Nevertheless, its co-inheritance presumably exacerbates PGK1-deficient phenotype, most likely due to a synergistic interaction of the affected genes both involved in cell energy supply.

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Introduction

There is an intriguing group of apparently balanced familial chromosomal translocations characterized by phenotypic inconsistency among carriers of cytogenetically similar rearrangements.

The most fascinating explanation for this would be the co-inheritance of the chromosomal translocation with a mutation at the same or at a distinct genetic *locus*, leading to a recessive monogenic or digenic disorder. Such a case

has not yet been described so far (Schäffer 2013). On the contrary, genomic imbalance at breakpoint regions is the most plausible explanation of such phenotypic inconsistency observed among carriers of similar chromosomal translocations (De Gregori et al. 2007).

Presently, the lack of a fully annotated human genome, including a haploinsufficiency map, hinders predictability of the phenotypic consequences of balanced chromosomal translocations even when the breakpoints are identified at nucleotide resolution. Although these may considerably affect the genomic architecture at breakpoint regions, a large majority are expected to be phenotypically imperceptible or subclinical. Nevertheless, otherwise subclinical translocations may modulate clinical phenotypes.

In eukaryotes ATP synthesis relies on glycolysis and oxidative phosphorylation (OXPHOS). Phosphoglycerate kinase 1 (PGK1; ATP: 3-phosphoglycerate 1-phosphotransferase; EC 2.7.2.3) is a multifunctional protein with a key role in ATP generation during glycolysis. PGK1 deficiency (OMIM #300653) is an X-linked recessive condition characterized by variable clinical manifestations involving up to three different tissues, i.e., red blood cells (RBC), skeletal muscle, and neurological tissue. However, patients rarely show all three clinical features (Beutler 2007; Chiarelli et al. 2012). To date, 22 different mutations have been identified in *PGK1*, and 17 of them have been characterized at the protein level (Chiarelli et al. 2012; Fermo et al. 2012; Tamai et al. 2014).

Mitochondrial complex I (CI) or NADH-ubiquinone reductase (EC 1.6.5.3) is part of the electron transport chain that results in ATP synthesis through complex V or ATP synthase of the OXPHOS pathway (Fassone and Rahman 2012). At least 12 assembly factors are required for the proper assembly, stability, or maturation of this CI (Fassone and Rahman 2012). One of these assembly factors is NUBPL, the depletion of which causes CI deficiency (OMIM #252010) (Tucker et al. 2012; Kevelam et al. 2013). Incorporation of Fe/S clusters into CI subunits by NUBPL is indispensable for electron transfer activity of CI (Sheftel et al. 2009).

Here we present detailed molecular and functional analyses demonstrating that the phenotype allegedly associated with the chromosome rearrangement t(3;14)(q26.33;q12) is actually due to a novel PGK1 variant, p.E120K, resulting in severe decrease of enzyme activity and that 14q12 breakpoint disruption of mitochondrial CI assembly factor, NUBPL, presumably exacerbates the neuromuscular symptoms of the enzyme deficiency.

Materials and Methods

Samples, Cytogenetic Analysis, and Lymphoblastoid Cell Lines (LCLs)

The family under study is from Hungary. Blood samples were collected after informed consent; the study was carried out according to the Principles of the Declaration of Helsinki of the World Medical Association.

Cytogenetic analyses and the establishment of LCLs were performed as previously described (David et al. 2009).

DNA extraction, flow sorting of derivative (der) chromosomes, array painting, amplification of the junction fragments, and SNP 6.0 array analysis

Genomic DNA extractions, flow sorting, and amplification of der chromosome-specific DNAs were performed as described earlier (David et al. 2009, 2013). Genomic amplicons of der(3) and der(14) chromosomes were analyzed by array painting using CytoScan HD array (Affymetrix, Santa Clara, CA, USA). The der(3) and der(14) junction fragments were amplified (see Table S1 for conditions) and sequenced by Sanger sequencing.

Genomic DNA from the index subject and his brothers (III:3 and III:6) was analyzed by Genome-Wide Human SNP 6.0 array (Affymetrix). Genotype calling was carried out using Genotyping Console software, and data was visualized using the Chromosome Analysis Suite (ChAS) software (Affymetrix).

Whole-Exome Sequencing (WES)

Exonic targets were captured and enriched using the SureSelect Human All Exon 50 Mb Kit from Agilent Technologies (Santa Clara, CA, USA) and sequenced on a HiSeq2000 instrument (Illumina, San Diego, CA) following the manufacturer's instructions. Variants were annotated, and known SNPs as well as novel genetic alterations were identified.

RNA Extraction and Expression Studies

Extraction of RNA samples from LCLs and reverse transcription (RT) reactions were performed as described earlier (David et al. 2013). For real-time quantitative PCR (RT-qPCR), see supplementary material.

Expression profiling of LCLs from the index subject and III:3 was carried out using the Human Gene 1.0 ST expression array (Affymetrix), and data analysis was performed as previously described (David et al. 2013).

Construction of the Expression Vector Coding for PGK1 p.E120K Variant

The vector encoding PGK1-E120K variant was obtained by subjecting pMM1 to site-directed mutagenesis using Quick Change XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) (Chiarelli et al. 2012). Mutagenic oligonucleotides are available in supplementary material.

Expression, Purification, and Characterization of the PGK1-E120K Variant

The PGK1-E120K was expressed in *E. coli* BL21(DE3) pLysS cells grown on ZYP-5052 autoinducing medium. The expressed enzyme variant was purified essentially as previously described (Chiarelli et al. 2012). Enzymatic activity and kinetic and thermal stability analyses have been performed (see the supplementary material).

SDS-PAGE and Immunodetection

SDS-PAGE analysis was performed in control and subjects' LCL's pellets (15 µg protein). For immunodetection the following antibodies were used: rabbit anti-NUBPL (Mitosciences, Eugene, OR, USA), mouse anti-tubulin (Sigma, St. Louis, USA), and MitoProfile Total OXPHOS antibody cocktail (Mitosciences, Eugene, OR, USA). Quantification was performed using the Quantity One (BioRad) software.

Results

Clinical Report

The first reported symptom of the index subject (Fig. 1, III:5) was neonatal hyperbilirubinemia. Transfusion therapy was necessary at the age of eight months because of severe anemia, jaundice, and hepatomegaly, leading to the diagnosis of idiopathic hemolytic anemia. His dysmorphic features resembled those of his affected brother III:4 (Supplementary Material and Table S2), but, additionally, brachycephaly, short neck, reduced length of phalanges, four-digit crease on the right palm, cutaneous syndactyly II/III of the feet, and severe hypotonia were also reported (Fig. S2C). Repeated therapy-resistant seizures started at one year of age, and his unconscious stage resulted in a suspected but unconfirmed diagnosis of meningoencephalitis. Besides epilepsy, one of the hemolytic events caused stroke and hemiparesis of the left side. Cognitive and somatic development of the child stopped at the age of one year and 6 months: he did not walk and could not speak, but he had no hearing loss. Swallowing difficulties became more

and more pronounced; gastrostoma and tube feeding were initiated from the age of 2 years. Severe recurrent hemolytic crises led to a partial splenectomy at the age of four and a half years. Positron emission tomography revealed Rasmussen's encephalitis at 5 years of age. At 6 years of age, ketogenic diet was initiated.

At the age of thirteen years, in 2013, he presented total body muscle atrophy, generalized somatic hypotrophy, diminished joint movements, muscle contractures, severe somato-mental retardation, no speech and no personal contact with other individuals, multiple dental caries, and bilateral cryptorchidism. The eyes are "swimming."

Two of his brothers (Fig. 1, III:3 and III:6) are healthy. In general, psychomotor development of III:3 was normal. He did not present any dysmorphic features. Reportedly, with physical effort, he shows signs of weakness and premature fatigue, well before his age-matched coworkers (Table 1).

Both parents are healthy, but the mother (II:5), in addition to anemia during pregnancies, also presents signs of fatigue.

During the course of this study, a maternal male cousin (Fig. 1, III:2), who died after falling down the stairs at the age of 7 years, was reported to have symptoms similar to his cousins.

His mother (II:3) and the remaining relatives (I:2, II:1, III:1) are all healthy. For additional clinical description, including subjects III:4 and III:2, see supplementary material.

Cytogenetic Analysis

Cytogenetic analysis of GTL-banded metaphase chromosomes performed on subject III:4 revealed an apparently balanced reciprocal chromosome translocation between the long arms of chromosomes 3 and 14 [46,XY,t(3;14)(q26.3;q12)] (Fig. S2A and B). Furthermore, his healthy mother (II:5) and two of his brothers (III:3 and III:5) were also carriers of a similar translocation (Fig. 1a).

No perfect co-segregation between the translocation and the reported phenotype could be observed; therefore, we hypothesized that an additional genomic or genetic defect might also segregate in this family.

Breakpoints and Candidate Genes

Genomic amplicons of flow-sorted der chromosomes were analyzed by array painting, and the breakpoints were narrowed to few kb regions (data not shown). Primers flanking the predicted breakpoints were designed, and the junction fragments were amplified and sequenced (Table S1). The alterations revealed by sequence alignments of the junction fragments are summarized in Fig. S1.

Table 1 Clinical features in subject carriers of either the *PGK1* c.358A mutation or the t(3;14) translocation alone or in association

Clinical features	III:2 <i>PGK1</i> c.358A	III:4 <i>PGK1</i> c.358A&t(3;14)	III:5 <i>PGK1</i> c.358A&t(3;14)	III:3 t(3;14)	NUBPL ^a 252010	CCDC39 ^a 613807	DNAJC19 ^a 610198
Hemolytic anemia	Severe	Severe	Severe	No	NR	NR	Normochromic microcytic
Erythroblastosis fetalis	Yes	Yes	Yes	No	–	–	NR
Jaundice	Yes	Yes	Yes	No	–	–	NR
Hyperbilirubinemia	Yes	Yes	Yes	No	–	–	NR
Hemolytic crisis	Severe	Severe	Severe	No	–	–	NR
Hepatosplenomegaly	Yes	Yes	Yes	No	–	–	NR
Splenectomy	ND	No	Yes	No	–	–	NR
Hemoglobin	Decreased	Decreased	Decreased	No	–	–	NR
MCV	Normal	Decreased	Decreased	No	–	–	Decreased
Neurological dysfunction	Yes	Yes	Yes	No	Characteristic	NR	Described
Neonatal hypotonia	No	Yes	Yes	No	–	–	NR
Developmental delay	Moderate	Yes	Yes	No	Characteristic	–	Described
Intellectual disability	Mild	Severe	Severe	No	Described	–	Described
Speech	Delayed	Absent	Absent	Normal	Described	–	Reported
Seizures, epilepsy	Severe	Severe	Severe	No	Reported	–	NR
Encephalopathy	Yes	Yes	Yes	No	Described	–	NR
Ataxia	NR	NR	NR	Transient	Described	–	Reported
Myopathy	Yes	Yes	Yes	No	Characteristic	NR	Cardiomyopathy
Muscular dystrophy	No	Severe	Severe	No	–	–	–
Weakness	Yes	Yes	Yes	Yes	Yes	–	Yes
Unsupported walking	Walk with help	Never sit or walk	Never walked	No	Characteristic	–	Characteristic
Spastic muscular tone	Yes	Yes	Yes	No	Described	–	–
Nuchal and dorsal muscle hypotonia	Yes	Yes	Yes	No	–	–	–
Swallow, tube feeding	Yes	Yes	Yes	No	Described	–	–
Horizontal nystagmus	Unknown	Yes	Yes	No	Characteristic	–	Reported
Infection	Repeated	Repeated	Repeated	No	–	Characteristic	–

^a OMIM number

NR not reported (see also Table S2)

The 3q26.33 breakpoint is at position g.180,278,286_180,278,294delTTCTGCA, 41.6 kb upstream of the 5' region of tetratricopeptide repeat domain 14 (*TTC14*), a gene whose function is presently unknown (Fig. S3). Additional genes localized further distal from the breakpoint are (1) the coiled-coil domain containing 39 (*CCDC39*) localized only at 53.5 kb, (2) the *FXR1*, and (3) the DnaJ (Hsp40) homologue, subfamily C, member 19

(*DNAJC19*). This gene encodes an important protein for mitochondrial physiology. Located at the inner mitochondrial membrane, it has been shown to function as a chaperone for mitochondrial protein import system. Defects in this gene are reported to cause an autosomal recessive secondary 3-methylglutaconic aciduria (OMIM #610198), a disorder that partially overlaps with that of reported subjects (Table 1) (Wortmann et al. 2012, 2013;

Fig. 1 (continued) Pedigree of the family, WES, and confirmation of the X-linked *PGK1* c.358G>A, p.E120K mutation. **(a)** Pedigree illustrating the segregation of the t(3;14)(q26.3;q12) and *PGK1* mutation. The index patient is indicated by *asterisk*. Translocation carriers are shown as hatched or half-hatched symbols, whereas filled and half-filled symbols denote the segregation of *PGK1* mutation.

Data in parentheses are inferred. The ratio of X-chromosome inactivation of *PGK1* mutation carrier females is indicated (Xa: active X chromosome). **(b)** Alignment of WES reads visualized using IGV tool indicating the homozygous single base substitution in exon 4 of *PGK1*. **(c, d)** Confirmation of the missense mutation c.358G>A; p.E120K by Sanger sequencing in subjects II:5 and III:5

Fig. 2 Overview of the chromosome 14q12 breakpoint region. (a) Schematic ideogram of chromosome 14; the breakpoint region is highlighted by a box. (b) Array analysis of the 14q12 breakpoint region using the Genome-Wide Human SNP array 6.0. A dashed line highlights the position of the breakpoint; Log2 ratios and allele differences are shown from the region (each dot point represents an oligonucleotide probe). Below, genes within this interval are depicted.

No genomic imbalance can be identified within this region. (c) Detailed physical map across the breakpoint region. Horizontal lines with folded gray arrows indicate the position of genes in sense (above the map) and antisense (below the map) orientation. (d) Detailed map of *NUBPL* (RefSeq NM_025152.2) indicating the position of breakpoint at nucleotide resolution and the positions of TaqMan gene expression assay probe

Davey et al. 2006). Therefore, serum and urine amino acid and urine organic acid levels were determined (see Table S3A and S3B). In subject III:3, 3-hydroxybutyric acid was especially increased, but also 3-methylglutaconic acid (3-MGA) and 3-methylglutaric acid (3-MG) were slightly increased.

The 14q12 breakpoint is at position g.32,248,943–32,248,944dupATAAGATAACAAG, within IVS6 of *NUBPL* (Fig. 2), a member of the Mrp/NBP35 ATP-binding protein family, and critical for the assembly of human respiratory mitochondrial CI (Sheftel et al. 2009). Recently, recessive mutations in *NUBPL* have been implicated in the genetic etiology of CI deficiency [OMIM #252010], a mitochondrial respiratory chain defect that is caused by mutations in multiple different nuclear, mitochondrial, or X-linked genes (Calvo et al. 2010; Fassone and Rahman 2012). Additionally, in subjects with pediatric

neurological disorders, three deletions encompassing this gene were reported ($p = 0.135$) (Cooper et al. 2011).

Exclusion of Genomic Imbalance and Identification of the Additional Genetic Defect

Pathogenic genomic imbalance that could explain the phenotypic inconsistency observed among translocation carriers was excluded by SNP 6.0 array analysis.

Subsequently, reevaluation of the family history revealed that the maternal cousin (III:2; Fig. 1a) presented a clinical phenotype resembling that of index subject. Based on this, two possible disorders, X-linked PGK1 and triosephosphate isomerase 1 (*TPI1*; OMIM #190450, 12p13.31) deficiencies, were suggested. Furthermore, X-chromosome segregation analysis among siblings using array-based SNP data corroborated the possibility of PGK1 deficiency (data not shown).

Consequently, WES of the index subject revealed a novel missense mutation c.358G>A that leads to a change from glutamic acid to lysine at position 120 of PGK1 (p.E120K) within exon 4 (Fig. 1b). Segregation study confirmed this alteration in the index subject and showed that female subjects I:2, II:3, and II:5 are asymptomatic carriers (Fig. 1a, c and d), whereas the remaining family members are normal. Substitution of the highly conserved, solvent-exposed, negatively charged glutamic acid 120 by positively charged lysine (E120K; Table S7, Figs. S4 and S7) increases the net charge of the protein by two units (from 2.8 to 4.8 at pH 7.4) and certainly strongly argues in favor of this being the disease-causing mutation.

Borderline skewed X-chromosome inactivation toward the normal allele (25:75) was observed in female II:5, the only mutation carrier that presented symptoms during pregnancy (data not shown).

Functional Characterization of the PGK1 p.E120K Variant

The specific activity of the purified PGK1-E120K variant was 10.2 U/mg compared to that of 816 U/mg of PGK1-WT (Chiarelli et al. 2012).

Kinetic analysis showed that, like PGK1-WT, PGK1-E120K variant was activated by high substrate concentrations (Fig. 3a and b) (Chiarelli et al. 2012). The mutant enzyme turned out to be severely affected in its kinetic properties (Table S4), showing a reduction of two orders of magnitude of its apparent k_{cat} values toward both substrates. The apparent K_m value toward 3-PG was sevenfold increased with respect to that of PGK1-WT, whereas that toward Mg-ATP was practically unchanged.

Interestingly, the variant's thermal inactivation rate curve was biphasic, with an increased slope after 5 min incubation, suggesting that it underwent heat-induced molecular change leading to the loss of enzyme activity (Fig. S5).

Gene Expression Studies

Expression array profiling of LCLs from translocation carriers (only one of them affected by the *PGK1* mutation) did not reveal significantly altered expression of genes from the breakpoints (Table S5). The expression level of the disrupted *NUBPL* was only reduced by ~1 SD.

Subsequently, expression levels of three of these genes were also analyzed in subjects II:5, III:5, III:3, III:6, and controls by RT-qPCR (Fig. 4a, Table S5, Fig. S6A and B). By this analysis, a statistically significant 2.6-fold reduction of the *NUBPL* expression level ($P = 0.0001$; 0.061 vs. 0.156) was observed.

Additionally, systematic analysis of array expression data of mitochondrial CI subunits did not show considerable alteration in their expression (Table S6).

SDS-PAGE Western Blot Analysis

Levels of the five OXPHOS complexes as well as the NUBPL assembly factor were determined by SDS-PAGE Western blot in LCLs, as our attempt to establish a fibroblast cell line from the index subject was unsuccessful.

NUBPL levels were reduced in all translocation carriers although at different extent (Fig. 4b). No significant changes were observed in the levels of the five complexes

Fig. 3 Kinetic profile of PGK1-E120K variant vs. wild-type enzyme. Steady-state kinetics of PGK1-E120K variant and wild-type as function of (a) Mg-ATP at fixed 5 mM 3-PG concentration and (b) as of 3-PG at fixed 5 mM Mg-ATP concentration. Open circles represent the PGK1-E120K variant, whereas filled circles represent

the wild type. Like the wild type, the PGK1-E120K variant presents a biphasic kinetic behavior toward the two substrates; therefore, the linear region (0–5 mM substrates, range of physiological concentration) was considered to extrapolate the kinetic parameters

Fig. 4 Quantification of *NUBPL* mRNA and protein in LCLs of translocation carriers and controls. **(a)** Bar graph depicting the relative expression of the *NUBPL* transcript comprising the exon 8–9 junction. Expression levels were normalized to the internal control (*DIDO1*). Fold change relative to reference is given in $2\Delta Ct$. Error bars represent standard deviation, and C1 to C13 are control LCLs from

unrelated subjects. **(b)** SDS-PAGE Western blot of *NUBPL* and alpha tubulin (loading control) in the family members and control LCLs showing significant reduction of *NUBPL* in the individuals affected by the translocation. *NUBPL* expression levels were normalized to the loading control. **(c)** SDS-PAGE Western blot of MitoProfile Total OXPHOS Human WB antibody cocktail

by the OXPHOS antibody cocktail (Fig. 4c). The dissimilar reduction of *NUBPL* protein in translocation carriers is in striking contrast with the evenly reduced mRNAs levels (Fig. 4a).

Discussion

Alleged association of the familial translocation t(3;14) (q26;q12) with a particularly severe hemolytic anemia and neuromyopathy led us to map the translocation breakpoints, at nucleotide resolution.

Given the absence of genomic imbalance that could explain the phenotypic inconsistency observed among translocation carriers, and taking into account previously unreported family medical history data, the presence of an additional genetic defect was hypothesized. Segregation analysis of X chromosome and WES revealed that the index subject is also affected by a novel X-linked *PGK1* mutation (c.358G>A, p.E120K) within a highly conserved region.

Biochemical and functional characterization of the *PGK1*-E120K variant showed highly perturbed kinetic properties (Table S4). Very likely the p.E120K substitution

leads to a distorted local protein structure with detrimental indirect effect on the active site geometry, locking the enzyme in a more tight conformation and hindering proper domain movements required for its catalytic activity. Additionally, the variant turned out to have weakened protein stability (Fig. S4) most likely due to an intrinsic propensity of aggregating in a temperature- and time-dependent manner (Pey et al. 2013). These data led us to conclude that p.E120K is one of the most severely impaired PGK1 variants characterized thus far (Fermo et al. 2012; Chiarelli et al. 2012) and consequently the main genetic cause of the phenotype observed in this family.

Although rare, lethal forms of PGK1 deficiency during infancy have been reported in the literature (Beutler 2007). Even more infrequent are PGK1-deficient patients exhibiting the three distinguishing clinical features of this deficiency (hereditary non-spherocytic hemolytic anemia, neurological dysfunction, and myopathy). In the family reported here, all affected subjects (III:2, III:4, and III:5) presented the full clinical spectrum of PGK1 deficiency, and seemingly they are among the most severely affected PGK1-deficient patients reported to date.

The disruption of *NUBPL* by the 14q12 breakpoint led to a 60% reduction of the mRNA expression level of this CI assembly factor in three family members. Concomitant reduction of the protein level was also observed, being the index subject, the most affected one. The translocation per se is only associated with a subclinical state as the only clinical feature reported in translocation carriers (without PGK1 deficiency) is exercise intolerance due to premature fatigue, a common symptom of mitochondrial myopathy.

Interestingly, homozygous subjects for the branch-site mutation c.815-27T>C within IVS 9 of *NUBPL* are predicted to have ~30% of *NUBPL* expression. This mutation was excluded in the presently reported family (data not shown). The partially impaired CI function in these subjects is also sub-pathologic and certainly not associated to a mitochondrial CI disease (Tucker et al. 2012).

Although generally the clinical phenotype of family members carrying identical PGK1 mutations is reportedly similar, both translocation carriers (III:4 and III:5), unlike their translocation noncarrier cousin, show severe neuromyopathy with exacerbated muscular dystrophy.

In different tissues or pathogenic conditions, one of the two metabolic pathways, glycolysis or OXPHOS, is predominant over the other (Hu et al. 2012). Additionally, these pathways are interconnected as cytosolic NADH, the electron supplier that drives ATP synthesis via OXPHOS, is generated through glycolysis to pyruvate by glyceraldehyde-

3-phosphate dehydrogenase (GAPDH) [see Glycolysis: Regulating Blood Glucose, <http://themedicalbiochemistry-page.org/glycolysis.php>] (Ramzan et al. 2013). Synergistic interactions, called synergistic heterozygosity, between defects in one or more different energy metabolism pathways have been reported as a common disease mechanism (Vockley et al. 2000). Such interaction between the reported genetic defects, both participating in cellular energy metabolism, seems obvious.

Although unlikely, we cannot exclude that other genes from the breakpoint regions, such as *DNAJC19*, may also contribute to the increased clinical severity of PGK1 deficiency in translocation carriers. The slight increase of both 3-MGA and 3-MG observed in the index subject can be related with several features of the clinical phenotype (metabolic disorder, mitochondrial dysfunction, progressive neuromuscular degeneration, and anemia) (Wortmann et al. 2013). Additionally, glycogen granules and granular structures in the mitochondria of endothelial cells and muscle fibers were reported in a PGK1-deficient subject (Schröder et al. 1996). Therefore, involvement of PGK1 deficiency in mitochondrial dysfunction seems likely and can also be related to the drastic reduction of *NUBPL* in the index subject.

Even more unlikely, but theoretically possible, is that the observed phenotype is exclusively the result of the novel PGK1 mutation, and the phenotype heterogeneity observed among translocation carriers and noncarriers is the outcome of each individual's genomic/epigenetic background.

In conclusion, the discordant inheritance of the allegedly translocation-associated severe phenotype, instead of being due to a genomic imbalance, is mainly explained by independent inheritance of a novel *PGK1* c.358G>A, p. E120K mutation. The pathogenicity of this mutation was confirmed by in vitro functional characterization of the variant. Furthermore, the t(3;14)(q26;q12) is only associated with a subclinical phenotype; nevertheless, disruption *NUBPL*, a component of the OXPHOS cellular energy pathway, most likely exacerbates the PGK1-deficient phenotype in subjects who are also translocation carriers. In addition, the case presented here constitutes a “beautiful” example of synergistic heterozygosity and a naturally occurring model to study the interactions between cellular energy pathways and their association with mitochondrial dysfunction. Therefore, further studies are warranted to clarify these additional aspects.

Additionally our data imply that synergistic interactions, involving heterozygous genomic and chromosome rearrangements, may contribute for the “missing” heritability

of inborn metabolic disorders. Last but not least, this study eloquently illustrates that even in our genomic era with WES and personalized genomics, a thorough family medical history is necessary in the elucidation of the molecular bases of rare diseases and their prevention.

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Synopsis

An extremely severe non-spherocytic hemolytic anemia and neuromyopathy, hypothesized to be associated with chromosome translocation-associated genomic imbalance, were shown to be mainly caused by a novel pathogenic severe *PGK1* mutation and that the neuromyopathic symptoms of the deficiency are likely exacerbated by the disruption of mitochondrial complex I assembly factor, NUBPL.

Compliance with Ethics Guidelines

Conflict of Interest

Dezső David, Lígia S. Almeida, Maristella Maggi, Carlos Araújo, Stefan Imreh, Giovanna Valentini, György Fekete, and Irén Haltrich declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

Informed consent was obtained from all subjects for being included in the study.

Additional informed consent was obtained for the patient for which identifying information (photograph) is included in this article.

Details of the Contributions of Individual Authors

DD designed, oriented, and analyzed the results and wrote the manuscript, LSA performed immunodetection of NUBPL, MM and GV performed in vitro characterization of the PGK1 variant, CA carried out RT-qPCR and sequencing of the junction fragments, SI contributed to the WES, and GF and IH contributed with identification, clinical description, and samples from family members. All authors contributed to the manuscript.

References

- Beutler E (2007) PGK deficiency. *Br J Haematol* 136:3–11
- Calvo SE, Tucker EJ, Compton AG et al (2010) High-throughput, pooled sequencing identifies mutations in NUBPL and FOXRED1 in human complex I deficiency. *Nat Genet* 42: 851–858
- Chiarelli LR, Morera SM, Bianchi P, Fermo E, Zanella A, Galizzi A, Valentini G (2012) Molecular insights on pathogenic effects of mutations causing phosphoglycerate kinase deficiency. *PLoS One* 7:e32065
- Cooper GM, Coe BP, Girirajan S (2011) A copy number variation morbidity map of developmental delay. *Nat Genet* 43:838–846
- Davey KM, Parboosingh JS, McLeod DR et al (2006) Mutation of DNAJC19, a human homologue of yeast inner mitochondrial membrane co-chaperones, causes DCMA syndrome, a novel autosomal recessive Barth syndrome-like condition. *J Med Genet* 43:385–393
- David D, Marques B, Ferreira C, Vieira P, Corona-Rivera A, Ferreira JC, van Bokhoven H (2009) Characterization of two ectrodactyly-associated translocation breakpoints separated by 2.5 Mb on chromosome 2q14.1-q14.2. *Eur J Hum Genet* 17:1024–1033
- David D, Marques B, Ferreira C et al (2013) A familial t(8;13) translocation associated TRPS I and a novel expanded TRP syndrome most likely caused by cis-rupture and increased *TRPS1* expression. *Hum Genet* 132:1287–1299
- De Gregori M, Ciccone R, Magini P et al (2007) Cryptic deletions are a common finding in “balanced” reciprocal and complex chromosome rearrangements: a study of 59 patients. *J Med Genet* 44: 750–762
- Fassone E, Rahman S (2012) Complex I deficiency: clinical features, biochemistry and molecular genetics. *J Med Genet* 49: 578–590
- Fermo E, Bianchi P, Chiarelli LR et al (2012) A new variant of phosphoglycerate kinase deficiency (p.I371K) with multiple tissue involvement: molecular and functional characterization. *Mol Genet Metab* 106:455–461
- Hu Y, Lu W, Chen G et al (2012) K-rasG12V transformation leads to mitochondrial dysfunction and a metabolic switch from oxidative phosphorylation to glycolysis. *Cell Res* 22:399–412
- Kevelam SH, Rodenburg RJ, Wolf NI et al (2013) NUBPL mutations in patients with complex I deficiency and a distinct MRI pattern. *Neurology* 80:1577–1583

- Pey AL, Mesa-Torres N, Chiarelli LR, Valentini G (2013) Structural and energetic basis of protein kinetic destabilization in human phosphoglycerate kinase 1 deficiency. *Biochemistry* 52: 1160–1170
- Ramzan R, Weber P, Linne U, Vogt S (2013) GAPDH: the missing link between glycolysis and mitochondrial oxidative phosphorylation? *Biochem Soc Trans* 41:1294–1297
- Schäffer AA (2013) Digenic inheritance in medical genetics. *J Med Genet* 50:641–652
- Schröder JM, Dodel R, Weis J, Stefanidis I, Reichmann H (1996) Mitochondrial changes in muscle phosphoglycerate kinase deficiency. *Clin Neuropathol* 15:34–40
- Sheftel AD, Stehling O, Pierik AJ et al (2009) Human Ind1, an iron-sulfur cluster assembly factor for respiratory complex I. *Mol Cell Biol* 29:6059–6073
- Tamai M, Kawano T, Saito R, Sakurai et al (2014) Phosphoglycerate kinase deficiency due to a novel mutation (c. 1180A>G) manifesting as chronic hemolytic anemia in a Japanese boy. *Int J Hematol* 100:393–397
- Tucker EJ, Mimaki M, Compton AG, McKenzie M, Ryan MT, Thorburn DR (2012) Next-generation sequencing in molecular diagnosis: NUBPL mutations highlight the challenges of variant detection and interpretation. *Hum Mutat* 33:411–418
- Vockley J, Rinaldo P, Bennett MJ, Matern D, Vladutiu GD (2000) Synergistic heterozygosity: disease resulting from multiple partial defects in one or more metabolic pathways. *Mol Genet Metab* 71: 10–18
- Wortmann SB, Kluijtmans LA, Engelke UFH et al (2012) The 3-methylglutaconic acidurias: what's new? *J Inherit Metab Dis* 35: 13–22
- Wortmann SB, Kluijtmans LA, Rodenburg RJ, et al (2013) 3-Methylglutaconic aciduria—lessons from 50 genes and 977 patients. *J Inherit Metab Dis* 36:913–921