Possible association of 3′ UTR +357 A>G, IVS11-nt 93 T>C, c.1311 C>T polymorphism with G6PD deficiency

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ABSTRACT

Background: Glucose-6-phosphate dehydrogenase (G6PD) deficiency is a common X-linked inherited enzymopathic disorder affecting more than 500 million people worldwide. It has so far been linked to 217 distinct genetic variants in the exons and exon–intron boundaries of the G6PD gene, giving rise to a wide range of biochemical heterogeneity and clinical manifestations.

Objectives: Reports from different settings suggested the association of intronic and other mutations outside the reading frame of the G6PD gene with reduced enzyme activity and presenting clinical symptoms. The present study aimed to investigate any association of other variations apart of the exonic or exonic intronic boundaries in the development of G6PD deficiency.

Methods: Sixty-seven unrelated Palestinian children admitted to the pediatric hospital with hemolytic crises due to G6PD deficiency were studied.

Results: In our Palestinian cohort of 67 [59 males (M) and 8 females (F)] G6PD-deficient children, previously hospitalized for acute hemolytic anemia due to favism, molecular sequencing of the G6PD gene revealed four cases (3M and 1F) that did not have any of the variants known to cause G6PD deficiency, but the 3′ UTR c.*+357 A>G (rs1050757) polymorphism in association with IVS11 (c.1365-13T>C; rs2071429), and c.1311 C>T (rs2230037).

Conclusion: We now provide an additional evidence from Palestinian G6PD-deficient subjects for a possible role of 3′ UTR c.*+357 A>G, c.1365-13T>C, and/or c.1311 C>T polymorphism for G6PD deficiency, suggesting that not only a single variation in the exonic or exonic intronic boundaries, but also a haplotype of G6PD should considered as a cause for G6PD deficiency.

KEYWORDS

Glucose-6-phosphate dehydrogenase (G6PD) deficiency; Gaza Palestine; 3′ UTR +357 A>G; IVS11-nt 93 T>C; c.1311 C>T; polymorphism

Introduction

Affecting more than 500 million people worldwide, glucose-6-phosphate dehydrogenase (G6PD) deficiency is a common X-linked (cytogenetic location: Xq28) inherited enzymopathic disorder which has been reported in peoples from nearly all geographical locations; however predominates where Plasmodium falciparum malaria is or had been endemic. The G6PD plays a crucial part in sustaining the integrity of red blood cells (RBC) by preventing the oxidation of hemoglobin and other cellular proteins through the reducing power of NADPH generated by G6PD in the hexose monophosphate shunt (pentose phosphate pathway). G6PD deficiency is associated with 217 distinct genetic variants in the exons and exon-intron boundaries of the G6PD gene [1,2]. These genetic variants result in diminished stability and or activity giving rise to a wide range of biochemical heterogeneity and clinical manifestations. This disorder is classified into five categories based on enzyme activity and clinical presentations [3]. Class I variants encompass the severely deficient cases with chronic non-spherocytic hemolytic anemia, while class V variants cause increased G6PD activity. The principle clinical presentation of G6PD deficiency is an acute hemolytic anemia (AHA) triggered by an exogenous drug or ingestion of fava beans that may require transfusion in children.

Recent studies from different populations have addressed probable and potential associations between some intronic and non-coding region polymorphisms and the biochemical and clinical presentation of G6PD-deficient subjects [4–10]. In the present study we aim to explore the association of 3′ UTR c.*+357 A>G (rs1050757), c.1365-13T>C (rs2071429, previously referred as IVS11 T93C in literature), and c.1311 C>T (rs2230037) polymorphisms with the G6PD deficiency among G6PD-deficient Palestinian children.
Table 1. The genetic variants identified in G6PD-deficient 67 samples.

| Location          | Number   | cDNA nucleotide substitution | Codon (a.a. substitution) | Class | Endonuclease digestion |
|-------------------|----------|------------------------------|---------------------------|-------|------------------------|
| G6PD Mediterranean| Exon 6   | rs5030868                    | 563C>T                    | II    | MboI                  |
| G6PD A-           | Exon 4/Exon 5 | rs1050828/rs1050829         | 188 (Ser>Pro)             | III   | NlaIII                |
|                   |          |                              | 202 G>A                    |       |                       |
|                   |          |                              | 376 A>G                    |       |                       |
| G6PD Cairo        | Exon 5   | CM962574                     | 404A>C                     | II*   | FnuI                  |
| G6PD Chatham      | Exon 9   | rs5030869                    | 404A>C                     |       |                       |
| G6PD Beverly Hills| Exon 10  | rs137862321                  | 1003G>A                    |       |                       |
| G6PD Gaza         | Exon     |                              | 1160G>A                    | II    | AcI                   |
| c* +357 A>G       | 3’ UTR   | rs1050757                    | +357 A>G                   |       |                       |
| c.1365-13T>C      | Intron 11| rs2071429                    | 93 T>C                     |       |                       |
| c.1311 C>T        | Exon 11  | rs2230037                    | 1311 C>T                   |       |                       |

*Based on Reading et al. [13].

Patients and methods

The present study was performed in accordance with the ethical standards established in the Declarations of Helsinki and has been approved by the Helsinki Ethical Committee of the Palestinian Health Research Council. Based on medical records from Al Nasser Pediatric hospital we enrolled 67 (59 boys and 8 girls) G6PD-deficient children with the informed consent from their parents or guardians.

Red blood cell count and indices (RBC count, Hb, Hct, MCV, MCH, MCHC), G6PD quantitative enzymatic activity, and DNA extraction for molecular investigations were obtained from venous blood samples collected in K2-EDTA tubes. The activity of G6PD enzyme was assessed spectrophotometrically at 340 nm using the G6PDH Screening Test (Randox Laboratories, Ltd, Antrim, UK) according to the manufacturer instructions, and G6PD activity was expressed as IU/g Hb at 37°C [11]. Dried blood spots were prepared by spotting approximately 400 µL whole blood on Ahlstrom 226 grade new-born screening filter paper (ID Biological Systems, Greenville, SC, U.S.A). DNA was extracted and purified from the dried blood spots as previously described [12]. Hematological and biochemical analyses were performed at Al Nasser Pediatric Hospital laboratories, Gaza, Palestine. All molecular analyses were conducted at the University of Utah and the Associated Regional and University Pathologists (ARUP) Laboratories, Salt Lake City, Utah, USA. The DNA samples were genotyped by polymerase chain reaction (PCR) amplification of G6PD exons 4, 5, 6 and restriction fragment length polymorphism (RFLP) analysis for A- -202, A- -376, Cairo -404C, and Mediterranean -563 variants (Table 1) as described [2]. Restriction enzyme digest products were analyzed using the Qiaxcel DNA analyser Screening kit and BioCalculator software (Qiagen Sciences, Germantown, MD, U.S.A). Those DNA samples without a defined G6PD mutation by the PCR-RFLP analysis were further characterized by BigDye™ terminator cycle sequencing on a 3100 Genetic Analyzer (Life Technologies Corporation, Carlsbad, CA, U.S.A) and Mutation Surveyor (SoftGenetics, LLC, State College, PA, U.S.A) analysis of the sequencing data.

Results

Enzyme activity measurements and other indices were taken at the time of hospitalization and at least 4 months after a child’s last reported AHA episode to ensure the assessment would be at a steady-state representation of G6PD activity (Table 2). At time of hospitalization activity levels for the 67 children ranged from 0.75 to 5.43 IU/gHb (normal range: 6.7–20.5); Hb levels 5.2–7.7 g/dL (normal range: 10.5–12.7 g/dL); reticulocyte counts 108–264 × 109/L (normal range: 28.5–78.1 × 109/L); MCV, MCH, MCHC, Hct, and DNA extraction for molecular investigations were obtained from venous blood samples collected in K2-EDTA tubes. The activity of G6PD enzyme was assessed spectrophotometrically at 340 nm using the G6PDH Screening Test (Randox Laboratories, Ltd, Antrim, UK) according to the manufacturer instructions, and G6PD activity was expressed as IU/g Hb at 37°C [11]. Dried blood spots were prepared by spotting approximately 400 µL whole blood on Ahlstrom 226 grade new-born screening filter paper (ID Biological Systems, Greenville, SC, U.S.A). DNA was extracted and purified from the dried blood spots as previously described [12]. Hematological and biochemical analyses were performed at Al Nasser Pediatric Hospital laboratories, Gaza, Palestine. All molecular analyses were conducted at the University of Utah and the Associated Regional and University Pathologists (ARUP) Laboratories, Salt Lake City, Utah, USA. The DNA samples were genotyped by polymerase chain reaction (PCR) amplification of G6PD exons 4, 5, 6 and restriction fragment length polymorphism (RFLP) analysis for A- -202, A- -376, Cairo -404C, and Mediterranean -563 variants (Table 1) as described [2]. Restriction enzyme digest products were analyzed using the Qiaxcel DNA analyser Screening kit and BioCalculator software (Qiagen Sciences, Germantown, MD, U.S.A). Those DNA samples without a defined G6PD mutation by the PCR-RFLP analysis were further characterized by BigDye™ terminator cycle sequencing on a 3100 Genetic Analyzer (Life Technologies Corporation, Carlsbad, CA, U.S.A) and Mutation Surveyor (SoftGenetics, LLC, State College, PA, U.S.A) analysis of the sequencing data.

Discussion

G6PD deficiency is a common disorder caused by many genetic variants that account for the biochemical and clinical heterogeneity of the disorder. With some exceptions, such as in Gaza Palestinian population [2,13], a single defined variant is typically endemic within defined ethnic/racial groups [14].
Currently 217 genetic variants have been linked to the clinical manifestation of reduced or deficient G6PD enzyme activity, vast majority located in exons and only rarely in exon/intron boundaries that interfere with the splicing process of G6PD mRNA [1]. The functional consequences of genomic changes outside of these regions have not been adequately explored. In recent years there have been numerous papers that have associated low G6PD enzyme activity with the common G6PD c.1311C>T/c.1365-13T>C haplotype [4–10]. Jiang et al. identified 59/1004 G6PD-deficient subjects (confirmed by lower enzymatic activity in accordance with class III) that carried the c.1311C>T/c.1365-13T>C haplotype with no other mutation within G6PD. These authors noted that the c.1311C>T/c.1365-13T>C haplotype occurs at a high frequency (∼6%) in the Chinese population, but only a portion of these present with variable degrees of decreased G6PD activity. Wang et al. also reported similar findings in 5 of 27 Malaysian G6PD-deficient subjects with the c.1311C>T/c.1365-13T>C haplotype (in the absence of any exonic variants) who had enzyme activity levels 30–50% of normal consistent with WHO Class III G6PD deficiency. Amini et al. identified the c.1311C>T/c.1365-13T>C haplotype was common in the Malaysian Negrito population with enzyme activity between 10 and 60% of normal values, while cases lacking this haplotype had normal G6PD activity. Amini and Ismail in a cohort of Malaysian subjects looked outside the G6PD coding region and identified three SNP (rs112950723, rs111485003 and rs1050757) with potentially strong association with the c.1311C>T/c.1365-13T>C haplotype and low enzyme activity. Using in-silico studies the authors showed that rs10507457 is sufficient to repress G6PD transcription by altering mRNA translation or stability leading to variable degrees of enzyme deficiency.

We report four of 67 G6PD-deficient Palestinian children, previously hospitalized for AHA due to favism, lacking any known mutations to cause G6PD deficiency. At their hospital admission the four children presented with the phenotype typical for favism including anemia, hemoglobinuria reported by parents as dark urine, jaundice, and a history of fava bean ingestion associated with reduced G6PD enzyme activity [15]. Of these, three children carried the 3’UTRc.*+357A>G/c.1365-13T>C/c.1311C>T haplotype and one child carried the 3’UTRc.*+357A>G polymorphism. Correlation of enzyme activity levels for these four individuals to the remainder of the cohort at time of hospitalization and while at steady-state revealed significant differences between the two groups (Table 2). These four children maintained,

### Table 2. Biochemical and hematological parameters of the four G6PD-deficient patients with polymorphism as compared to 63 patients with exonic mutations.

| Patients | Genotype | Sex | Neonatal jaundice | Agea (months) | At admission of first hemolytic crisis | At steady state | Neonatal jaundice | Agea (months) |
|---------|----------|-----|-------------------|--------------|--------------------------------------|---------------|-------------------|--------------|
| 1       | C.* +357 A>G/c.1365-13T>C/c.1311C>T | M | Yes | 15 | 2.90 | 6.2 | 121.0 | 1.4 | 7.51 | 11.7 | 52.03 | 0.95 |
| 2       | C.* +357 A>G/c.1365-13T>C/c.1311C>T | M | Yes | 24 | 2.23 | 7.1 | 133.4 | 1.5 | 7.51 | 11.7 | 52.03 | 0.95 |
| 3       | C.* +357 A>G/c.1365-13T>C/c.1311C>T | F | No | 34 | 6.3 | 12.9 | 264.2 | 2.6 | 7.7 | 11.9 | 28.5 | 0.73 |
| 4       | C.* +357 A>G | M | No | 30 | 5.43 | 7.7 | 108.1 | 1.94 | 7.7 | 11.9 | 28.5 | 0.73 |
| 63 subjects | Mean | | | | | | | | | | | |

aAge at first hemolytic crisis required hospitalization.

bNormal values as mentioned in Reading et al. [13].

c3′UTRc.* +357A>G
although in the deficient range, higher enzyme levels during AHA crisis (3.69 IU/gHb average) and at steady-state (7.29 IU/gHb average, low end of normal range) than the 63 children carrying known G6PD deficiency mutations. The clinical presentation during AHA crisis was also milder for these four children with higher RBC indices and lower unconjugated bilirubin levels (Table 2), therefore, these polymorphic variants may be classified as class III in the G6PD enzyme activity scale [3]. We cannot exclude the possibility of other more rare enzymopathies (e.g. such as enzymes of glutathione synthesis or transport pathways or glutathione reductase deficiency) as the primary cause of phenotype [16] because the G6PD activity was reduced during the crises and in remained at the steady-state in the low end of the normal range after hemolytic episode.

In this study we presented supporting data and observations linking non-coding variants to the incidence of G6PD deficiency. We recognize the c.1311T>C/c.1365-15T>C haplotype is commonly associated with those G6PD genotypes (i.e. G6PD Mediterranean, Viangchan) that lead to G6PD deficiency and therefore should be noted in the molecular evaluation of G6PD deficiency. To this association we would now add the 3′UTR variant c.Δ+357A>G as an associated deficient genotype and categorize it as a WHO class III G6PD deficiency. Identifying the frequency of these polymorphic variants among the general population was not performed in the present study, and thus we recommend further studies to determine the frequency of these polymorphic variants not only in the healthy Palestinian population but also searching for these variants in G6PD-deficient patients from other populations especially when no mutations are detected in the 13 G6PD exons or exonic–intronic boundaries [1].

Disclosure statement

No potential conflict of interest was reported by the authors.

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