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Research Article

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A Novel 1421TG (KPK) Mutaion at Exon 11 and 563C/T Varient at Exon 6 Found from Local Population of Pakistan

Sadaf Naimat[1], Muhammad Mateen Tahir[2]*, Sabrina Waheed[3], Mehwish Siddique[3], Pakeeza Arzoo Shaiq[1], Gabriel Herrera López[4] and Muhammad Naseem Ullah[5]

[1]*University Institute of Biochemistry and Biotechnology, Pir Mehr Ali Shah, Arid Agriculture University, Rawalpindi, Pakistan*

[2]*Key Laboratory of Molecular Epigenetics, Ministry of Education and Institute of Cytology and Genetics, Northeast Normal University, Changchun, China*

[3]*Government Graduate College for Women Satellite town Gujranwala, Pakistan*

[4]*Biological and Environmental Science and Engineering Division King Abdullah University of Science and Technology Thuwal Saudi Arabia*

[5]*Department of Zoology, Sahiwal campus, Virtual University of Pakistan*

***Corresponding author:** Muhammad Mateen Tahir, Key Laboratory of Molecular Epigenetics, Ministry of Education and Institute of Cytology and Genetics, Northeast Normal University, Changchun, China

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Abstract

Introduction: More than 140 genetic variations exist for the most frequent inborn error of metabolism, glucose-6-phosphate dehydrogenase deficiency. In Pakistan, there is a 2–9% incidence of G6PD deficiency. In addition, Pakistani researchers have explored G6PD variations to some extent, which has been documented and made popular worldwide. Thus, the purpose of this work was to characterize the prevalence of new mutations, related enzyme activities, and G6PD variations 563C/T at (exon 6) and 1131 C/T polymorphism (exon 11) in Pakistan.

Methods: Individuals with a G6PD deficiency diagnosis were included. We used RFLP-PCR to find frequently occurring mutations that have been reported from Asian countries in the past. Moreover, exon (11) amplification was carried out, followed by gene sequencing. The quantitative enzyme assay was used to measure the activity of the G6PD enzyme.

Results: One hundred G6PD-deficient patients were examined, of which 96 were men and 4 were women. A mutant version of G6PD Mediterranean (563C-T) was also discovered. Furthermore, a novel mutation 1421 T>G was found in exon 11, resulting in the anticipated amino acid shift from glycine to valine. After the proband's place of origin, this was given the name G6PD Kpk. Both variations showed extremely low levels of G6PD enzyme activity, and they were also males who had been diagnosed as G6PD defective at birth. Two months passed in one, and five months in the other

Conclusion: Although the mutant variation (150 bp) we observed in our results was different from the natural 563C/T (which gives a band of 377,119), The most prevalent G6PD variation, in our opinion, was still 563C-T. Moreover, the unique genomic variation 1421T-G was found. Particularly low G6PD 563C-T and new G6PD enzyme activity was observed. In order to anticipate additional novelties and mutations in Pakistan, a considerable percentage of samples are needed for additional mutational investigation.

Keywords: 563C-T; Genetic mutation; Genomic variation; G6PD deficiency

Abbreviations: G6PD: Glucose-6-phosphate dehydrogenase; DCPIP: Dichlorophenolindophenol; EDTA: Ethylenediaminetetraacetic acid; NADP: Nicotinamide adenine dinucleotide; NADPH: Nicotinamide Adenine Dinucleotide Phosphate

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Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is a widespread disease that affects people worldwide. The oxidative stress caused by fava beans and infections can cause acute hemolytic anemia in those with G6PD deficiency due to their higher vulnerability to these events [1]. Moreover, neonates deficient in G6PD have an increased risk of jaundice, which can quickly evolve into bilirubin encephalopathy and result in kernicterus, a devastating neurological condition that is irreversible [2]. The G6PD gene, which codes for the G6PD enzyme, is found at the Xq28 band. It is roughly 18 kb long, with 13 exons and 12 introns. An X-linked incomplete recessive inheritance pattern is primarily responsible for the genetic alterations that lead to G6PD deficiency. These mutations frequently result in polymorphic variations that cause problems with protein folding and decreased enzyme activity (Pfeffer et al., 2022). Thus far, about 230 genetic variations that are clinically significant have been found [3]. Different ethnic groups and geographical regions have different rates of prevalence of G6PD deficiency [1,3].

The incidence in male Pakistanis ranges from 2 to 4%, with Pathans having a higher frequency—8%. Out of several newborn jaundice-related admissions, the study also showed from earlier research that 8% had a G6PD deficiency. Anemia patients' total incidence was found to be 3.4% in anemic individuals and 1.36% in a previous study. Incidence was found to be 1.8% in a sample of young individuals in good health, with the exception of Pathans, who showed no ethnic variability [4]. The most frequent variant in the Pakistani population, accounting for 78% of the sample, was G6PD Mediterranean, according to a molecular genetics article. G6PD Chatham and Orissa came in second and third, with 5% and 0.7% of the sample, respectively [4].

G6PD deficiency affects over 400 million people worldwide, with a prevalence of 4.9% [5, 6]. In countries where malaria is widespread, the incidence is considerably greater, at 8% [7]. The most common type of substitution is a single amino acid since this genetic condition has been linked to over 160 single nucleotide alterations at the DNA level [5]. Amino acid substitutions cause the G6PD enzyme to undergo a number of structural alterations, such as disruption of the protein structure, stabilization of the G6PD protein, deformation of the glucose-6-phosphate dehydrogenase active site, impairment of the NADP+ binding site, and alteration in the interface of monomer interaction, all of which reduce enzyme activity [8,9]. The purpose of this study was to examine the underlying genetic abnormalities that cause G6PD deficiency in Pakistani people. To find the mutations (563C/T and 1311C/T) in exons (6, 11) that caused a similar drop in enzyme activity, G6PD-deficient specimens were examined.

Materials and Methods

Subjects enrolled

A total of 96 males and 4 women were examined for G6PD gene mutations among the subjects enrolled in order to ascertain the prevalence and range of genetic variants in the Pakistani community. During our hospital visits, G6PD deficiency was identified in all patients, who were of Pakistani origin. The sample sources for the blood were the city's blood collection centers and equivalent facilities across the nation. A total of 100 G6PD-deficient participants were enrolled, representing five major ethnic groups and living in four different provinces across the nation. To identify G6PD-deficient participants and obtain their consent for research enrollment, a consent form was created specifically for this study.

G6PD assessment and complete blood counts

Each laboratory-based computerized data set yielded the results of the G6PD enzyme assays and complete blood count (conducted on a Coulter Gen-S; Coulter Electronics, Hialeah, FL, USA). A kit from Atlas Medical UK was used to measure the enzyme activity. Based on the idea that G6PD reduces NADP to create NADPH by acting as a substrate on glucose 6 phosphate, the test was designed. This leads to the reduction of the colorful substrate dichlorophenol indophenol blue to a colorless form (DCPIP). The rate at which the substrate decolored was directly proportional to the enzyme concentration in the sample. Decolonization periods were recorded after thorough mixing and incubation. Those that were deficient had longer than two to twenty-four hours, and those who were normal had decolonization times between five to sixty minutes. Samples with positive results. Additional genetic and biochemical analysis was performed on each laboratory-based sample that tested positive (deficient), based on the results of the G6PD enzyme assays and CBC report (conducted on a Coulter Gen-S; Coulter Electronics, Hialeah, FL, USA). Hemoglobin concentration was used to determine how much blood was needed for the test (Table 1).

Table 1: This table depicts the amount of blood required according to HB (hemoglobin concentration g/dl) level of individual used a screening kit (Atlas Medical UK kit).

Hemoglobin Concentration g/dl	Quantity of blood required ml
7 – 9.5	0.04
9.6 – 11.5	0.03
11.6 – 13.5	0.025
13.5 – 15.0	0.02

Molecular studies

Five milliliters of EDTA blood were drawn from all individual using a modified DNA extraction technique, and the white cells were separated for DNA extraction by centrifuging the blood. It was maintained at -80°C in a frozen state until additional work was done. To examine our samples, we used a two-step approach. Considering that reports of G6PD Mediterranean in the Pakistani population [10-12]. Considering previously published methods, RFLP-PCR was first applied to all samples' DNA in order to identify 563 C-T mutations [13]. Using the primer and enzyme sets listed in Table 2, we conducted a PCR analysis to investigate exon 6 of 563C/T. Each PCR reaction was comprised of a 25µl volume, 200 ng of isolated genomic DNA, each dNTP (0.2Mm) Taq polymerase (2U), MgCl2 (1.5Mm), Tris-HCl (PH (8.8);34Mm), ammonium sulfate (8.3 mM), MgCl2(0.2 mM) that was extracted. Using Mbo II restriction enzymes, the PCR products were digested overnight at 37°C. This was done in 30 cycles, which consisted of 1 minute at 94°C, 30 seconds at 58/62°C (563 C-T), and 40 seconds at 72°C. The digested products were then subjected to an electrophoretic analysis.

Table 2: G6PD variant that underwent RFLP-PCR screening during the research process.

Primers use according reference	Exon	Nucleotide substitution	G6PD variant	Amino acid substitution	PCR size bp		
					Wild	Variant	Enzyme
Saha *et al*. (1994) [10]	6	563C-T	Mediterranean	ser188phe	377,119	150	MboII

1311C/T polymorphism (exon 11)

Amplification of exon 11 (fragment size both forward and reverse; 160 bp) was also performed using the dideoxy technique to examine C/T variations of G6PD other than exon 6 in undigested samples from Pakistan. In each 20ul PCR product, the following components were included: 10ul red Solis bio-Dye master mix; 1ul forward and reverse primer; 6ul Betaine; 2ul DNA; and optimum thermal cycler conditions (Tables 3,4).

Table 3: Ingredient mixture of PCR for sequencing.

Ingredients	Volume (Ul)
Red Solis bio Dyne master mix	10ul
Forward primer	1ul
Reverse primer	1ul
Betaine	6ul
DNA	2ul

Table 4: Thermo cycler conditions.

PCR Conditions Cycle	Temperature		Time	Cycle
Initial Denaturation	95		5 minutes	1
Denaturation	95		30 seconds	34
Annealing	Exon	Temperature	60 seconds	
	11	65		
Extension	72		60 seconds	
Final extension	72		5 minutes	1
Storage	4			

Sequence analysis

Products from the PCR were transported to the University of CMB in Lahore. Bioinformatics software and tools like as NCBI, Bio Edit, and Chroma exe were used to examine these data. From ensemble (ensemble.org/index.html) the reference sequence was acquired.

Ethical issues

All subjects gave their informed consent before being enrolled in the study, which was authorized by the institutional ethical review committee.

Results

For their Mediterranean mutation, 96 men and 4 women in all

were assessed. G6PD Mediterranean samples from Mbo II yielded 277, 119, and 110 bp fragments, while normal patients showed 377 and 119 bp fragments (Table 2). 150 bp of a new variation was discovered in place of them (Figure 1). As shown in Figure 1, Lanes 1–7, the amplified product (exon 6) containing 264 bp was digested using Mbo11. In comparison, a 150 bp band was observed (lane 3) together with a 264 bp band after Mbo11 digestion of the PCR generated fragment from a G6PD Mediterranean sample. The G6PD gene's exon 6 has a C-T mutation at position 563, which is consistent with these alterations. There was one male observation and none for females. Consequently, males exhibited a comparatively higher frequency of G6PD Mediterranean at exon 6.

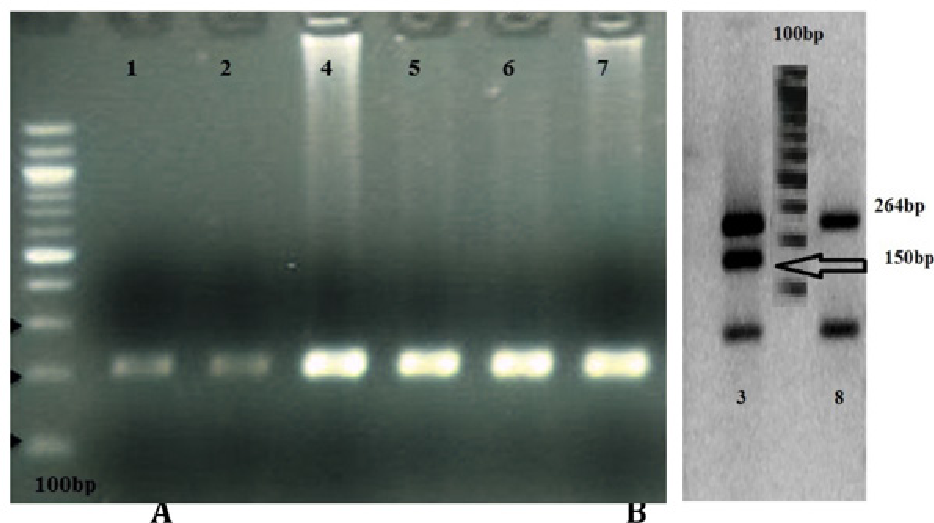

Figure 1: Mediterranean mutations. Analysis of amplified G6PD DNA fragments for the 563 C-T (Mediterranean) mutation. Size marker, G6PD patients digested with Mbo11 (fig A, B lane 1 and 2) are depicted. Also shown are digested fragments from a G6PD Mediterranean positive and negative (B, A. lane 1, 1-7).

Detection of 1311C/T mutation at Eon 11

In order to find the 1311C/T Polymorphism in exon 11, Sanger sequencing was carried out on 100 G6PD defective DNA samples. Software (Chromas exe, Ensemble, and bio-edit) was used to analyze the data. Rather than the predicted 1311C/T a novel mutation T>G was discovered. Additional research revealed the substitution of the amino acid glycine for valine; the cDNA location was (p.V474G) Figure 2. Polyphen-2 and the Sift score revealed a detrimental effect on the protein structure of the G6PD enzyme through further research using molecular docking methods to examine its effects on enzyme structure Figures 3 & 4.

Discussion

The 1311 C/T polymorphism and 563 C/T of the G6PD gene were investigated in detail in the Pakistani population with G6PD deficiency. Despite the fact that this polymorphism is frequently found in individuals who carry the G6PD Mediterranean gene, we found the variant in our sample in addition to the Mediterranean variant, not the wild type. Due to the diverse ethnic groups residing in Pakistan, we were able to evaluate both through the investigation and comprehend the genetic basis of G6PD deficiency in the Pakistani population and the previously reported mutations 1311C/T [4] and 563C/T in the word wild [10]. Two separate mutations were discovered in this investigation: a variant (563 C-T) and a novel mutation (T>G), which was discovered for the first time in Pakistan. Through data analysis and sequencing with technologies that led to the finding of mutations, a new mutation, G6PD in 1421 T>G KPK, was discovered (Figures 2-4).

X-linked illness is, predictably, widespread in Pakistan. It only showed a general trend toward marriages between relatives, with half of the women marrying close biological relatives [14]. Differential phenotypes can be produced by either preferentially selecting the flawed clone or X-chromosome inactivation that is not random, according to Kaplan et al. (1999) [15]. In carriers' red blood cells, G6PD activity can therefore be significantly decreased, intermediate, or normal. Their low levels of enzyme activity could contribute to the explanation of the 563C-T mutant variant's detection. An amino acid substitution (188 SerfiPhe) has been identified in exon 6 of G6PD Mediterranean (563C-T) Type II [8] varieties are widely distributed in Southern Europe [16-18], and African countries [19,20]. G6PD Mediterranean was connected to extremely little activity of enzyme despite the fact that individuals were identified during vigorous hemolysis; this attribute is congruent with results that have been previously described [21]. The low enzyme activity < 1U/gHb of the predicted 563C/T mutation was confirmed by PCR-RFLP analysis. Despite disparities in cultural backgrounds, we hypothesized that this molecular homogeneity could be the result of enduring genetic connections or shared ancestry among different cultural groups.

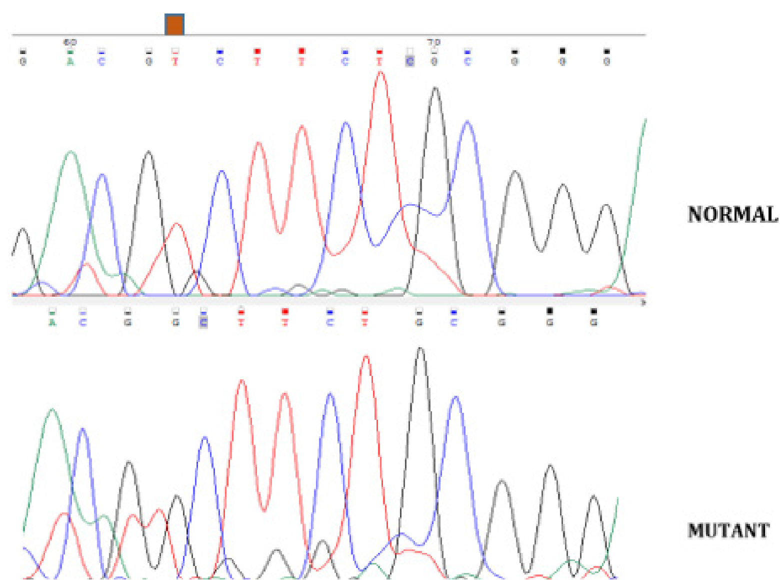

Figure 2: Novel mutation of G6PD gene at exon 11 showed the substitution of T>G, which changed the whole amino acid i.e. valine into glycine. Orange box indicated the base substitution in normal and mutant individual.

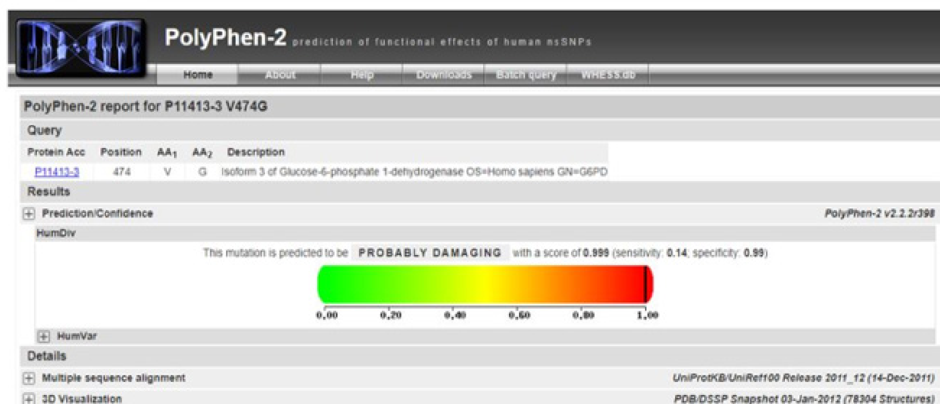

Figure 3: The above figure was depicted that the novel mutation have to probably damaging effect with a score of 0.99 touched to 1.00 which is damaging lie under red bar with sensitivity (0.14) and specificity (0.99) by using bioinformatics software called polyphen-2.

User Input	ENSP	Pos	Ref	Subst	Prediction	SIFT Score	Median Information Content	# Seqs
ENSP00000377192,V474G	ENSP00000377192	474	V	G	DAMAGING	0	2.57	266

Figure 4: The above figure shows the sift score of predicted novel mutation at exon 11have sift score 0, which means that its effect on G6PD gene is damaging.

Vulliamy et al. (1988) reported that the Mediterranean mutation is linked to a silent polymorphism (C/T) at nucleotide 1311 [8]. Although the variant located at location 1311 is in discordance with G6PD Mediterranean, it is not a faulty allele in and of itself. At first, this was noted in Middle Eastern and European nations [18,21]. However, additional reports from the Indian subcontinent were received later (Sukumar et al., 2004). According to Moiz et al. (2013[5]) [4], the high frequency of 32% was seen in those with normal g6PD activity. The Pakistani population exhibits an intriguing mixed 1311 T polymorphism, which may indicate a recombinant occurrence. We found that exon 11 of the 1311T polymorphism is variable. The intent of my research was to assess the variants for exons 6 and 11 of the G6PD gene, which have already been reported in previous studies, in the Pakistani population. During this time, we identified a 1:1 mutation in both exons in 100 samples, and at 2 and 5 months of age, we were able to distinguish the babies as boys rather than girls, suggesting that G6PD is more common in men than women. It consequently indicated that extensive investigation may reveal substantial variations within the Pakistani population. Although certain variations, including 563C/T and 1311C/T, are known to be prevalent in the community, none of these were found in my investigation; instead, I discovered novel 150bp Mediterranean variants and G6PD KPK (exon 6).

Conclusion

Till now, there has been little knowledge about the mutations responsible for G6PD deficiency in Pakistan. The unique genomic variation 1421T-G was also found. Particularly low G6PD 563C-T and new G6PD enzyme activity were observed. In order to anticipate additional novel mutations in Pakistan, a significant percentage of samples will be required for additional mutational testing.

Conflict of Interest

None.

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