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IDENTIFICATION OF GLUCOSE 6 PHOSPHATE DEHYDROGENASE MUTATIONS BY SINGLE STRAND CONFORMATION POLYMORPHISM AND GENE SEQUENCING ANALYSIS
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IDENTIFICATION OF GLUCOSE 6 PHOSPHATE DEHYDROGENASE MUTATIONS BY SINGLE STRAND CONFORMATION POLYMORPHISM AND GENE SEQUENCING ANALYSIS

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ABSTRACT

***Objective*: To identify mutation among Turkish individuals who demonstrated deficiency of glucose 6 phosphate dehydrogenase(G 6 P D).**
***Design*: Laboratory based experimental study.**
***Setting*: The molecular diagnostic laboratory of the Royal Postgraduate medical school Hammersmith Hospital, London**
***Subject*: Six DNA samples from Turkish males confirmed to have G-6-PD deficiency where available for the study.**
***Results*: One subject was found to have an abnormal mobility shift on SSCP in exon 9 and subsequent direct gene sequencing of exon 9 revealed a single base change at position 1003 G>A. This causes an amino acid substitution Alanine > Threonine at position 335: This mutation is G6PD chatham. All the other 5 DNA samples did not show any abnormal mobility shift on SSCP**
***Conclusion*: The stepwise PCR-SSCP and direct gene sequencing is a faster and more convenient method of identifying mutations. In this study the yield was low. Only one of the six Turkish DNA samples confirmed as G6PD deficient showed abnormal mobility shift on SSCP and was identified as G6PD Chatham on gene sequencing.**

INTRODUCTION

Gliucose-6-phospate dehydrogenase (G6PD) is an enzyme that is present in the cytoplasm of all cells where it plays a key role in glucose metabolism. G6PD deficiency is the most common inherited disorder in man and it is estimated to affect about 400 million people worldwide (1). The kurdish Jews have the highest prevalence in the world with 70% of male affected (2). In Japan and Northern Europe G6PD deficiency is rare and the frequency is 0.1% (3). Most G6PD deficient individuals are asymptomatic and only develop symptoms in responses to oxidant stress when they develop acute haemalytic anaemia. The main precipitating agents are drugs, infections and ingestions of fava beans

The human G6PD gene was cloned and sequenced in 1986 and full genomic structure elucidated about the same time(4,5). The gene encoding G6PD maps on the telomeric region of the long arm of X-chrosome (bandXq 28).The genomic DNA of G6PD spans about 18kb and consists of 13 Exons and 12 introns

The human X-linked genetic locus encoding G6PD is highly polymorphic and 160 mutations have been determined (6). The mutations are mainly misense mutations causing amino acid substitutions and are scattered throughout the coding exons. Only three deletions have so far been reported with the largest showing eight base pair deletions. Larger deletions and other mutations such as nonsense mutations and fremeshift mutations that could completely abolish the function of the proteins have not been found in G6PD gene. This is probably because complete absence of this gene is incompatible with life.

Polymorphic G6PD variants (also known as WHO CLASS II and CLASS III) are those that have realised appreciable gene frequencies (1-70%) in particular populations(7). It is now established that the high frequency of polymorphic variances has arisen because G6PD deficiency gives relative protection against malaria . Chronic nonspherocytic haemolytic anemia (also known as WHO CLASS I) occur at very low frequencies in any part of the world(8).

The main clinical presentations in G6PD deficiency include neonatal jaudice, acute haemolytic anaemia and chronic nonspherocytic haemolytic anaemia. Most people with polymorphic G6PD gene never suffer any clinical manifestations and the variant causing chronic haemolysis are extremely rare with a frequency of 1 in 10^6(9). The complications of G6PD deficiency can be greatly reduced by screening newborns in regions with high frequency of G6PD

deficiency and by educating the population to avoid the drugs and fava beans that precipitate attacks of hemolytic anemia.

MATERIALS AND METHODS

Six DNA samples from male Turkish individuals were available for the study. These were from individuals known to have G6PD deficiency and who had been previously studied for common mutations G6PD Mediterranean, Seattle, A- and Aures using PCR and restrictions enzyme digestion method but failed to show these mutations.

The samples tested were first amplified from genomic DNA using polymerase chain reaction as described by salki R *et al*(10).For each reaction the constuents were as follows.

PCR buffer	5ul
DMSO	5ul
dNTP	o.6ul
Primer 1(100ng/ul)	1ul
Primer2(100ng/ul)	1ul
Sterile water	36ul
Taq polymerse(1U/ul)	1ul
DNA	1ul

The above was mixed and put in a PCR machine. After denaturing the DNA at 94 C for ten minutes the machine performed 30 cycles of

1 minute at 94 C	denaturing
1 minute at 56C	annealing
1 minute at 72C	extension

SSCP analysis: The method of analysis was that described by Orite *at el* (12). The PCR reaction was scaled to a final volume of 25ul.The entire coding sequence was amplified in 8 fragments (exons 2,3 and 4,5,6 and 7,8,9,10 and 11-13) by using 8 pairs of primers.

Fragments were labeled by including a trace of a P32 of d CTP during amplification.1ul of DNA was added to each reaction. A proportion of each amplified sample (5ul) was mixed with equal volume of formammide dye and heated to 95° for 5 minutes and was snap frozen in dry icebath. 4 ul of each was loaded on 6% polyacrylamide gel containing 0.5X Trisborose buffer and 10% glycerol.

The gel was run overnight at 4mA(275v) at room temperature. It was run until xylene cynol had reached about 30cm from origin. The gel was dried on whatman paper and exposed to X- ray film over night.

Direct gene sequencing: In the SSCP analysis whenever an abnormal mobility shift was detected, the corresponding genomic regions were amplified further and investigated by direct genomic sequencing as described by S Thein S.L *et al* (12) The segments of the genomic DNA to be sequenced was amplified using the PCR method as described but with the following modifications. The volume for each sample was increased to 100ul and one of the primers was biotinylated at its 5′ end.

Generation of single stranded DNA: After making sure that the amplification was successful, 50ul dynabeads M- stepavid (dynabi UK) was added. The beads were first washed twice with Tris EDTA sodium hydroxide(TES) using dynal magnetic separater E (MPC-E) and were then added to PCR products and left on the bench for 5 minutes.The supernatant was then removed using the MPC-E, The single stranded biotinylated template which now have been immobilised by the dynabeads were washed once with TES fo,, owed 100ul sterile water using MPC-E and lastly suspended in 7ul of water.

DNA sequencing: The sequencing reactions were performed by dideozaytermination method using sequencing kit (USB). Either the non-brotinglated primer or the internal primer was used for this. After completion of the reaction and addition of termination of solution, the reaction was heated for 5 minutes. The supernatant which contains the newly synthesed DNA strands was separated using MPC-E. 2.5ul of each was electrophoresed in a 6% polyacrylamide gelfor two hours. The gel was fixed in 10% acetic acid and dried on Whatman paper. after overnight exposure to x-ray film the sequence was read.

RESULTS

All the DNA samples were subjected to SSCP analysis. The entire coding region was amplified in eight fragments .
Subject 5 showed an abnormal mobility shift in exon 9 Subjects 1 ,2,3,4,and 6 did not show any abnormal moblity shift.

Results of gene sequencing analysis: DNA from subject 5 showed a mutation in exon 9 at position 1003 of a single base change G > A. This mutation results to a change in amino acid sequence Alanine > Threonine at position 335.This mutations is G-6-PD chatam and has been described before.

Gene sequence analysis for part of exon 9 from subjects (Graphic Presentations).

DISCUSSION

One subject was found to have an abnormal mobility shift on SSCP in Exon 9. Subsequent direct gene sequencing of exon 9 revealed a single base change at position 1003 G > A. This causes an amino acid

substitution, Alanine to threonine at position 335. This mutation is G6PD Chatham. This mutation was first described in a boy of Indian ancestry living in England who had neonatal jaundice. Since then the mutation had been described in an Algerian (13) and a Greek subject (14).

It is apparent that this mutation is found in populations of different ethnic groups and raises the question of common ancestry among these population groups. This question can be answered by conducting a hyptotype study.

SSCP mutation analysis has proved to have a high resolving power provided the fragment is not more than 300 base pair. The resolving power of SSCP tends to decrease with increasing length of amplified fragments.

In some cases a band shift has not been seen in a fragment that is as small as 216 base pairs. This might be due to the fact that the mutation are very close to the end of restriction fragment involved (15). Hence both the position of the mutation and the size of the amplified fragment can affect the resolving power SSCP.

PCR - SSCP is a most sensitive technique in analyzing mutations and has been extensively used. Judging from the experience of different groups PCR - SSCP technique is able to detect up to 90% of single base mutations in fragments of 200 base pair (16)
In this study the yield from SSCP was low. Only one of six DNA samples confirmed as G6PD deficient showed an abnormal mobility shift in SSCP and a mutation G6PD Chatam identified.

Overall the stepwise PCR-SSCP and direct gene sequencing is certainly a faster and convenient method of identifying undetermined mutations.

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