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Molecular Characterization of New Variants of Glucose-6-phosphate Dehydrogenase Deficiency Gene Isolated in Western Province of Saudi Arabia Causing Hemolytic Anemia

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Abstract: In order to explore the polymorphism of G6PD deficiency in Western region of KSA, 49 unrelated Saudis aged from 5 days to 15 years with G6PD-deficiency (41 males and 8 females) were investigated genetically by using the polymerase chain reaction, PCR-restriction enzyme (PCR-RE) analysis combined with single-strand conformational polymorphism (PCR-SSCP) analysis and direct PCR-sequencing procedure to identify the different G6PD deficiency variants. Ten different missense mutations were detected, eight of them have been reported as previously known G6PD variants in addition to two new novel mutations, in this screening. The following mutations were found: G6PD Mediterranean 563^{C-T} (twenty two cases; 45%); G6PD A^{-} {12 cases contains; G6PD Matera $202^{A-G}/376^{A-G}$ (ten cases; 20.4%), G6PD Betica $968^{T-C}/376^{A-G}$ (two cases; 4.1%)}, G6PD Aures 143^{C-T} (six case; 12.2%), G6PD Nara (three cases; 6.1%); G6PD Chatam 1003^{G-A} (2 cases; 4.1%); G6PD Union 1360^{T-C} (one case; 2.0%) and Viangchan G6PD 871^{G-A} (one case; 2.0%). One of two remaining patients showed a novel base substitution mutation A to G transition at nucleotide position 1342 in exon 11 (1342^{A-G}) which has not been reported in any other ethnic group. This mutation results in a (448) Ser to Gly substitution and the resulting G6PD variant was designated as G6PD Haweia. This new mutation creates a *Hae*III recognition site which digested a 497 bp band into two bands of sizes 307 and 190 bp by using PCR-RE analysis. Other novel mutation type is three base deletions at position 516-518 located in exon 6 and resulted in Gly 174 amino acid removal which describes as G6PD Taif. The biochemical features of these two mutations have not been characterized. The data indicate that G6PD Med^{+}, G6PDA^{-} and G6PD Aures mutations are the most common mutations (84%) in the Western province.

Key words: G6PD deficiency, base substitution, frameshift mutation, hemolytic anemia

INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD; E.C.1.1.1.49.) is a 'house keeping' enzyme which catalyses the first step in the hexose monophosphate pathway and provides a reductive potential, in the form of nicotinamide adenine dinucleotide phosphate (NADPH), required for a variety of biosynthetic reactions and for the detoxification reactions of free radicals and peroxides within the cells. In cells lacking mitochondria, such as mature red blood cells (RBCs) that have a relatively long survival time (3 months) and lack protein synthesis, hexose monophosphate shunt is the only process to generate NADPH. In which, glucose-6-phosphate (G6P) is converted into 6-phospho-gluconolactone, catalyzed by G6PD and accompanied by the reduction of NADP into NADPH. A sufficient amount of NADPH is essential for the integrity of RBCs, because NADPH reduces glutathione, which protects these cells against oxidative stress. Thus, G6PD protein instability is first manifested in RBCs (MacDonald *et al*., 1991; Maeda *et al*., 1992). G6PD deficiency therefore leads to neonatal jaundice and hemolytic anemia (Beutler, 1994, 1996), ranging from mild hemolytic anemia induced by ingestion of fava beans (favism), infections or drugs to chronic nonspherocytic anemia with attacks of severe anemia induced by infections or drugs (Beutler, 1991).

G6PD deficiency is one of the most common enzymopathies, which affects more than 400 million people worldwide (Beutler, 1993, 1996; Vulliamy *et al*., 1993). According to WHO criteria (Betke *et al*., 1967), so far over 442 different variants of G6PD have been identified by the standardized enzymological methods, grouped in five classes on the basis of their activity and clinical manifestations (Betke *et al*., 1967). G6PD deficiency usually becomes manifest in most cases in hemizygous males (Betke *et al*., 1967; Abdulrazzaq *et al*., 1999; Laosombata *et al*., 2005). The clinical presentations

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of the G6PD-deficient subjects of Asian origin appear to be different from those of African Americans with G6PD deficiency (Beutler, 1993). This may be due in part to the fact that most African Americans with G6PD deficiency are asymptomatic (Beutler, 1993). Approximately 20% to 40% of neonatal jaundice is related to G6PD deficiency in Taiwan (Chiu *et al*., 1993; Hirono *et al*., 2002). In contrast, neonatal jaundice has rarely been attributed to G6PD deficiency in the United States (Chiu *et al*., 1993).

The active G6PD enzyme exists as a dimmer (Beutler, 1993, 1996) and its monomer structure consists of 515 amino acid subunits with a calculated molecular weight of 59,256 daltons (Beutler, 1996). The G6PD gene is located in the q28 region of the X chromosome. It consists of 13 exons and 12 introns distributed over approx. 20 kb of genomic DNA (Beutler, 1993, 1994). The exons range in size from 38 bp (exon 3) to 695 bp (exon 13). All introns are smaller than 300 bp, except intron 2, which is about 11 kb in size. The coding region is in exons 2-13, while exon 1, the first 8 bp of exon 2 and the first 88 bp of exon 13 form the untranslated portions of the G6PD mRNA (Beutler, 1993). About 326 of G6PD deficiency have been studied genetically and more than 126 different mutants have been characterized by cloning the mutant genes using cDNA or genomic libraries (Betke *et al*., 1967). Most of the genetic studies of G6PD have been done on samples from African Americans (Beutler, 1991; Hirono *et al*., 1989) and people of Mediterranean ancestry (Vulliamy *et al*., 1993). The highest frequency values of G6PD deficiency are found in tropical Africa, in the Middle East, in some areas of the Mediterranean, in tropical and sub-tropical Asia (Hy *et al*., 2005). The DNA sequence of Asian G6PD variants has not been extensively investigated. Although there is a regional high frequency (2 to 16%) of G6PD deficiency in Taiwan and Southern China, in which 17 mutations were found only in Chinese (Chiu *et al*., 1991, 1993; Beutler, 1996). Recent molecular studies elucidated that most of the variant enzymes are produced by one or two missense mutations in the structural gene (Beutler, 1993; Hirono and Miwa, 1993). The clinical phenotype is variable but often predictable from the molecular lesion. Class I variants (the most severe forms of the disease) cluster within exon 10 in a region that is believed to be involved in dimerization at the protein level (Hirono and Miwa, 1993). The severity of the G6PD deficiency depends on the effects of the mutation on protein stability and activity (Betke *et al*., 1967; Beutler, 1996).

Interestingly, only few missense and small in-frame deletions are known. Therefore, most of the mutations identified so far are point mutations (96.8%) causing single amino acid substitutions in the G6PD gene (Vulliamy *et al*., 1993). The only, four exceptions out of 126 identified mutations (3.2%) are G6PD deletions for three bases or more. These deletions are Sunderland (MacDonald *et al*., 1991), which is associated with a 3 bp deletion, (CAT) that occurs in exon 2, resulting in the loss of an isoleucine residue at position 35 in the G6PD enzyme, G6PD Stonybrook (MacDonald *et al*., 1991; Hirono and Miwa, 1993) that is characterized with 6 bp deletion (GGCAAC) in exon 7 resulting in losing Glycine and Threonine amino acids at positions 242-243 in the G6PD enzyme, G6PD Nara (Hirono *et al*., 1993) represented 24 bp deletion in exon 9 at position 953-976 losing of 8 amino acids from position 319 to position 326 in the G6PD gene and G6PD Vancouver (Maeda *et al*., 1992), with three missense base substitution mutations in exon 6 at positions $317^{C\text{-}G}$, $544^{C\text{-}T}$ and $592^{C\text{-}T}$ resulting in three amino acid substitution (106 Ser-Cys, 182 Arg-Trp and 198 Arg-Cys) respectively, in the G6PD gene. Except for these cases, no mutations occur in the G6PD gene's promoter or splicing site regions and no other types of mutation, such as large deletions, or frameshift addition mutations, which would severely alter or completely abolish the protein's function were identified (Beutler, 1991; Maeda *et al*., 1992), this is probably because severely altered G6PD function is lethal.

There is a considerable amount of data on the incidence of G6PD deficiency in Arabian region and few of these studies had concerned the molecular basis of G6PD deficiency at genomic level. However, the Mediterranean mutations ($563^{C\text{-}T}$) being the major variant in this region (Bayourni *et al*., 1996; Samilchuk *et al*., 2003) results in the replacement of serine with phenylalanine at position 188, followed by A^{-} ($202^{G\text{-}A}$; Hirono *et al*., 2002), Aures ($143^{T\text{-}C}$) and Chatham ($1003^{G\text{-}A}$) as described in different studies from United Arab Emirates (UAE), Saudi Arabia, Oman and Kuwait (Beutler, 1991, 1993; Samilchuk *et al*., 2003). Cumulative data from different regions of Saudi Arabia proved that the rate of G6PD deficiency accompanied with neonatal hyperbilirubinemia with or without hemolytic anemia is relatively high ranged from 1.9% to about 18% (Ali *et al*., 2002; Muzaffer, 2005), specially in Southern regions with a very few molecular studies for characterization of such variants or identification of new types (Muzaffer, 2005). This study analysis the mutation of 49 different G6PD deficiency cases at molecular level in the coding sequence of the G6PD gene.

MATERIALS AND METHODS

Subjects: Forty nine unrelated patient (41 male and 8 female), aged between 5 days and 15 years, who were admitted to three National Hospital in KSA during

last three years (2002-2005). They had mild to acute hemolytic anemia and were diagnosed as a G6PD deficient cases. Fifteen milliliter of fresh blood samples were obtained from the subjects by veinpuncture and divided into two parts, 5 mL was mixed with acid citrate dextrose (ACD) for screening G6PD activity that was performed in the hospitals with a kit supplied by sigma as described by the manufacturer (Sigma, st Louis, MO). Another 10 mL of blood collected in EDTA for molecular studies.

Genomic DNA purification and DNA amplification: Total genomic DNA was isolated and purified from the peripheral blood leukocytes (the buffy coat) of 49 patients by standard phenol/chloroform extraction (Maniatis *et al*., 1982). PCR amplification of the entire coding sequence of G6PD gene was performed using eight sets of primers covering all the coding exons from this genomic DNA. The sequence of each of the 16 oligonucleotides and their position relative to the coding regions of the G6PD gene was carried out as described previously (Poggi *et al*., 1990a). The synthetic oligonucleotides were assembled on a DNA synthesizer (Applied Biosystems, Inc, Foster City, CA) and purified as described by Tang *et al.* (1990). The PCR reaction was carried out described previously (Saiki *et al*., 1988). The amplification reaction mixture contained 1.0 μg genomic DNA in 50 mmol L^{-1} Tris-HCl pH 8.3, 1.5 mmol L^{-1} $MgCl_2$, 200 pmol L^{-1} of each dNTPs, 200 ng of each primer and 1.0 U of Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). The reactions were performed on a DNA thermal cycler (Perkin-Elmer Cetus) as follows: One predenaturation cycle at 95°C for 10 min., followed by 35 cycles of denaturaton at 94°C for 1.0 min, annealing at 55°C for 1.5 min and elongation at 72°C for 2.0 min, finally
the reaction was incubated at 72°C for 7 min for final extension in a final volume of 50 μL.

Restriction fragment length polymorphisms (RFLPs) of PCR products: Twenty five microliter portion of each amplified fragments were extracted with phenol/chlorophorm, precipitated with 2.5 volume absolute cold ethanol and resuspended into 25 μL TE buffer. Five microliter of each DNA amplified fragment was digested with 10 units of *Mbo*II, *Mbo*I, *Fsb*I, *Fok*I, *Nla*III, *Xcm*I, *Msp*I, *Nla*IV, *Alu*I, *Ava*II and *Bcl*I *restriction* enzymes (New England Biolabs and Northumbria Biologicals) at 37°C for 48 h using buffers and conditions recommended by the manufacturers' instructions. Those restriction enzymes used for detection of the following point base substitution mutations (transversion, or transition); Mediterranean mutant (563^{T-C}), Aures (143^{T-C}), Union (1360^{C-T}), A^+ (376^{A-G}), three different variants of A^- (202^{G-A}; 968^{T-C}; 680^{G-T}), Viangchan (871^{G-A}), Mahidol (487^{G-A}), Chinese-3 (493^{A-G}) and a silent mutation 1311^{T-C}. The digested DNA fragments were gel-electrophoresed on 3.0% agarose gel, at 80 Volt for about 45 min, stained with ethidium bromide then visualized and photographed under UV light. The position of each mutation was expressed using the cDNA number (Poggi *et al*., 1990b).

PCR-based single-strand conformation polymorphism (PCR-SSCP) analysis: All the amplified different coding regions (exons) that not digested with any of previous restriction endnucleases were then submitted to SSCP analysis (Orita *et al*., 1989; Ainsworth *et al*., 1991) with modification of using a 8% polyacrylamide gel system and ethidium bromide staining (Orita *et al*., 1989; Yap and McGee, 1992; Hirono *et al*., 1995). Briefly, 3 μL of the amplified product was added to 27 μL of 92% formamide, 20 mmol L^{-1} EDTA and 0.05% bromophenol blue, denatured at 98°C for 10 min and quenched on ice for 2 min. Ten microliter of this mixture was loaded on a 8% polyacrylamide mini-gel (0.75 mm × 6×8 cm; acrylamide: bis 38:1.5), run at 150 volts and stained with ethidium bromide.

Subcloning and DNA sequencing: PCR amplified fragments that show negative digestion with or without different mobility shift (compared with normal DNA mobility) were subjected to phenol/chlorophorm extraction, precipitation by ethanol, then resuspended into 20 μL TE buffer and finally the DNA was purified on a 1.0% low-melting agarose gel (Seaplaque; FMC BioProducts, Rockland, ME). The purified DNA was ligated into pGEM3Zf(+) vector (TA kit; Promega-Biotech, Madison, WI) containing SP6 and T7 promoters flanking the cloning site. DNA sequencing of clones was performed by the dideoxynucleotide sequencing method (Sanger *et al*., 1977) using [^{35}S]dATP and 2.0 Sequenase kit (United States Biochemical Corp, Cleveland, OH, USA) and universal primers complementary to the flanking promoters sequences from both sides.

Haplotype analysis: Haplotype analysis was carried out on different amplified fragments for all samples using above mentioned 8 sets of primers and restriction endonucleases (*Bcl*I and *Fok*I) as previously described (Martinz *et al*., 1994).

Southern blotting: The PCR products were Southern-transferred to a nylon membrane (Amersham). The southern blot filter was hybridized to a radioactive labeled probe [^{32}P] of about 307 bp (higher band of *Hae*III

Table 1: Distribution of G6PD mutations among 49 G6PD deficient Saudis

Common mutation name	Mutation (cDNA position)	Patients No.	Patients (%)	Restriction endonuclease	Sequence	Amino acid replacement	Exon	*WHO type
Mediterranean	563^{C-T}	22	44.9	*Mob*II	TCC-TTC	188Ser-Phe	6	2
A-	$202^{G-A}/376^{A-G}$	10	20.4	NlaIII/FokI	GTG-ATG/	{126Asn-Asp/68Val-Met}		
	968^{T-C} /376^{A-G}	2	4.1	XcmI/FokI	CTG-CCG/	{323Leu-Pro/68Val-Met}	4	3
Aures	143^{T-C}	6	12.3	*Mob*I	ATC-ACC	481 Ile-Thr	2	2
Nara	953-976 deletion	3	6.1	-	24 bp deletion	319-326 del	9	1
Chatham	1003^{G-A}	2	4.1	*Hpa*II	GCC-ACC	335Ala-Thr	9	3
Union	1360^{C-T}	1	2.0	*Fsb*I	AAT-GAT	454Arg-Cys	11	2
viangchan	871^{G-A}	1	2.0	*Nla*IV	GTG-ATG	291Val-Met	7	2
New types	516-518 deletion	1	2.0	-	3 bp deletion	Del. 174 Gly	6	-
	1342^{A-G}	1	2.0	*Hae*III	ACC-GCC	448Ser-Gly	11	-
Total	-	49	100	-	-	-		-

* Class 1 nonspherolytic hemolytic anemia; class 2, severe deficient; class 3, moderate deficient

digestion of 497 bp fragment of mutant exon 10+11). Analysis of *HaeIII* digestion of PCR-DNA products was carried out by southern hybridization using conventional methods (Maniatis *et al*., 1982).

RESULTS

Molecular analysis of the G6PD variant gene: The strategy of G6PD gene analysis was firstly screening for the most ten common G6PD variants that had previously been reported among Southest Asia G6PD deficient individuals by PCR/RE method. Samples showing an absence of these ten mutations were subjected to PCR-SSCP analysis followed by direct DNA sequencing to characterize the mutations in the samples. All amplified DNA fragments were firstly subjected to direct PCR/RFLPs test for expected G6PD mutations. They were digested with *Mbo*II endonuclease to detect the 563^{C-T} mutation in exon 6 which is the most prevalent variant in Asia. Samples negative for *Mob*II digestion were further digested with *Mbo*I to test 143^{T-C} mutation and who were negative for *Mob*I subsequently digested with *Fsb*I to test G6PD union 1360^{C-T} mutation. All negative samples with previous digestion were subjected to digest with *Fok*I alone or combined with *Nla*III, *Xcm*I, *Msp*I, for testing A^+ or most predominant three different A^- mutations (202^{G-A}, 968^{T-C} and 680^{G-T}) respectively. Samples that didn't show any of identified digestion pattern were subjected to *Nla*IV, *Fsb*I, *Alu*I or *Ava*II digestion for detection of Viangchan, Union, Mahidoh, or Chinease-3 mutations respectively. All samples were further digested with *Bcl*I in order to detect another allele mutant with or without previous digestion. Indeed 22 samples out of 49 (45%) G6PD deficient mutations were found to have Mediterranean mutation, followed by A^- (12/49 samples; 24.5%); which classified into Matera; ($202^{G-A}/376^{A-G}$) as detected in 10/49 (20.4%) and two samples (Betica; $968^{T-C}/376^{A-G}$) represents 2.1%, while third mutation ($202^{G-A}/376^{A-G}$) was not detected at any patients under studying. The variant Aures was detected in 6/49 representing 12.2%, Union was detected in only one sample (2.0%) and Viangchan was found in one sample (2.0%). Other expected mutations were not found in this screening by PCR/RFLPs method (Table 1). Remaining seven samples were further analyzed by PCR-SSCP and/or direct sequencing of G6PD exons by using corresponding 8 sets of primers.

PCR-SSCP and sequence analysis: To search for other mutations in 7 samples negative for above RFLP tests, PCR-SSCP analysis was performed on amplified coding region of different amplified fragments. These samples showed three different abnormal patterns (pattern A, for three samples in exon 9, pattern B, for one sample in exon 6+7 and pattern C for three samples in exons 9 and 10+11). The four samples in pattern A (lanes 1-3) and B (lane 2) showed clear shorter mobility shifts on the gel, while pattern C didn't show any abnormal bands compared with normal subjects (Fig. 1a-c). The four samples with pattern A and B were submitted subsequently to sequence analysis, which revealed a frameshift deletion in all cases; three cases (3.1%) had the deletion of frameshift mutation 953-976 (G6PD Nara; Fig. 2a) in exon 9 and one new interesting mutation that not previously described or detected before which represent three base deletion in exon 6+7 at position 516-518 (GGG) that corresponding to Gly amino acid at position 174 of G6PD gene (Fig. 2b). Those deletions don't alter the reading frame and should produced a mutant protein eight and one amino acid(s) shorter respectively compared with the normal G6PD gene. Comparison of partial amino acid sequence of normal and mutated sequence was represented in Fig. (2a and b). *Bsy*I restriction enzyme recognition site was abolished due to deletion in Gly amino acid (Fig. 3) compared with normal G6PD gene of exon 6+7. On the other hand, three samples in

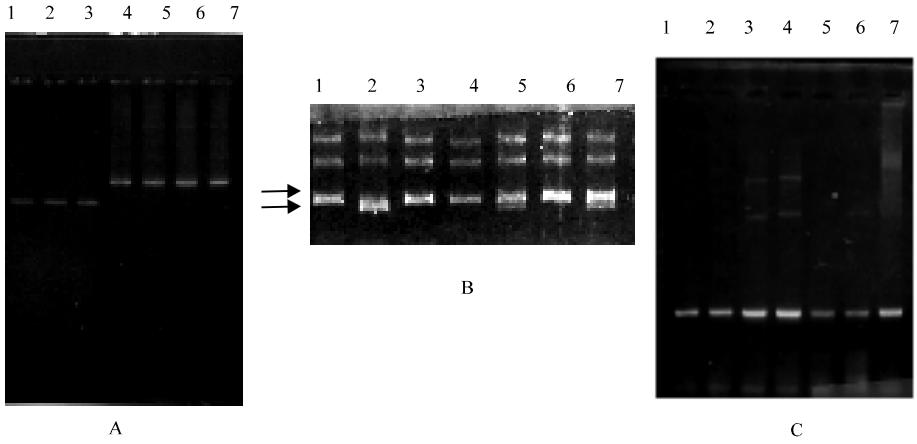

Fig. 1: PCR-SSCP analysis: panel A represents deletion in 24 nucleotides in exons 9; lanes 1-3 are G6PD Nara, lanes 4-7 normal PCR fragments, panel B is 3 nucleotides deletions in exon 6; lane 2 of G6PD variant sample, lanes 1, 3-7 are control samples without any deletions; panel C represents of base substitution mutation without any difference in mobility shift in exon 10+11 of G6PD gene

A- exon 9
Normal
313 D G E G E A **T K G Y L D D P** T V P 329
…GATGGAGAGGGCGAGG**CCAC**CAAAGGGTACCTGGACGACC**CCAC**GGTGCCCC…

G6PD Nara
313 D G E G E A T V P 321
…GATGGAGAGGGCGAGGCCCGGTGCCCC…

B- exon 6
Normal
171 K P F G R D L Q S S D R L S N H I S S L 190
… AAGCCCTTC**GGGAGGGA**CCTGCAGAGCTCTGACCGGCTGTCCAACCACATCTCCTCCCT…

G6PD Taif
171 K P F R D L Q S S D R L S N H I S S L 189
… AAGCCCTTCAGGGACCTGCAGAGCTCTGACCGGCTGTCCAACCACATCTCCTCCCT…

Fig. 2: A 24 nucleotide deletion in G6PD Nara gene (A) in exon 9 and G6PD Taif gene (B) in exon 6. The deletion occurred between two direct tetranucleotide repeats CCAC in case of Nara and GGGA in case of Taif

pattern C sequence analysis showed two cases (4.1%) with base transition mutations in exon 9 at base 1003^{G-A}, that previously reported as G6PD Catham (Beutler, 1991), which causes an Ala to Thr amino acid at position 335 and this G-A mutation abolished a *Fsp*I restriction endonuclease site where the normal samples were cut into two fragments but the mutant sample remains undigested and instead *Hpa*II was created. Again sequence analysis revealed one new base substitution mutation 1342^{A-G} in exon 10+11. The A-G transition mutation at position 1342 caused a Ser to Gly replacement at amino acid 448. This mutation creates a new site for *Hae*III restriction endonuclease, that was proved by digestion (PCR/RE) of 497 bp amplified PCR-fragment into two fragments of 190 and a 307 bp in the presence of this mutation (Fig. 4a). The result of all mutations analysis are summarized in Table 1.

Southern blot analysis: The existence of 1342^{A-G} transition mutation that creates *Hae*III restriction site was also confirmed as in Fig. 4b that showed the autoradiogram of 497 and 307 bp fragment by using a prob of 307 bp.

Haplotype analysis: It revealed that all 6 cases of Aures 143^{T-C}, 19 samples of Med^{+} 563^{C-T} and the case of Viangchan 871^{G-A} mutations were on chromosome with the

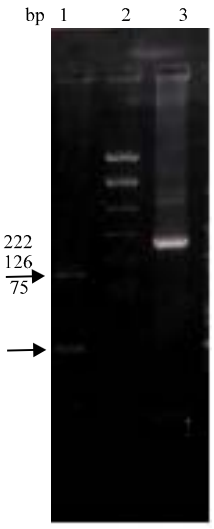

Fig. 3: New frameshift deletion mutation in 516-518 bp that abolished cleavages site, by *Bsy*I restriction enzyme at exon 6+7 in lane 3, lane 2 is molecular weight marker, lane 1 normal G6PD exon 6+7 with restriction site, G6PD

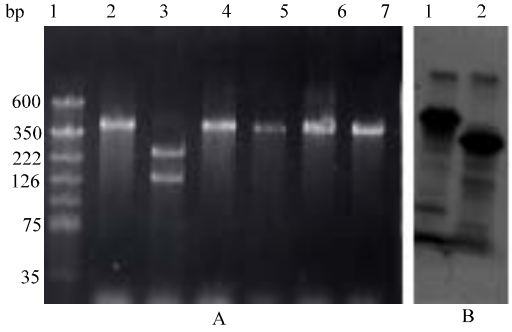

Fig. 4: New mutation in 1342 A G transition creates *Hae*III restriction site at exon 11, Lane 1 molecular weight marker, lanes 2, 4-7 normal undigested G6PD gene, lane 3 is digested HaeIII G6PD gene. Panel A ethedium bromide staining, panel B is autoradiography of lanes 2,3 with upper labeled fragment after *Hae*III digestion

1311^{T-C} alleles (*BclI*$^+$). However, the two samples for Chatham 1003^{G-A} mutations and all 12 A$^-$ variants had 1311^{T-C} negative alleles (*BclI*$^-$). The ten cases of the 202^{G-A} and the two cases of 968^{T-C} were on chromosomes with the 376^{A-G} allele (*FokI*$^+$) that gives different types of A$^-$ variants. The new base substitution mutation 1342^{A-G} was in chromosome had *BclI*$^+$ allele.

DISCUSSION

Identification of G6PD mutations: The prevalence of G6PD deficiency was varies greatly throughout the world. In some Asiatic populations, G6PD deficiency is known to exist with a relatively high frequency (Chiu *et al*., 1991; Martinz *et al*., 1994). In KSA the rate of G6PD deficiency characterized with neonatal hyperbilirubinemia and favism induced hemolytic anemia recently raised (Ali *et al*., 2002; Muzaffer, 2005) in certain regions. In the present study, the molecular defects of the G6PD gene have characterized in 49 Saudi patients with G6PD deficient in Western province. Eight variants have been identified as documented previously and two unique mutations were identified which clearly reflects a high heterogeneities of such mutations in this region. The most three prevalent types (Med$^+$, A$^-$ and Aures) of G6PD deficiency in this study represent 40/49 cases (81.6%), suggesting that these three alleles are common among Saudis with G6PD deficiency, while other seven different variant types showed low prevalence rate (9/49; 18.4%).

Base substitution mutation: The first type of mutation as expected is the Mediterranean mutation (563^{C-T}) which is the most prevalent variant that identified in 22 patients out of 49 (44.9%). In which single base change results in a 188 Alanine to Serine amino acid substitution (Med$^+$), that is reported to be one of the most common deficient variants in South East Asia and Mediterranean region (Beutler, 1991; Calabro *et al*., 1993; Cittadella *et al*., 1997). Pervious study on Kuwaiti chromosomes with the Mediterranean mutation (Calabro *et al*., 1993; Samilchuk *et al*., 2003) reported that all Med$^+$ patients were polymorphic 1311^{T-C} allele (*BclI*$^+$). Such a Med$^+$/*BclI*$^+$ haplotype is typical for the Mediterranean region and the Middle East, while most cases from India and South East Asia are Med$^+$/*BclI*$^-$, suggesting independent origin of Mediterranean mutations of two different haplotypes (Beutler, 1991; Chiu *et al*., 1991). Interestingly, only 19 cases of the 22 samples examined (86.4%) in this study have a substitution at 1311^{C-T} causing silent amino acid substitution (Med$^+$/*BclI*$^+$). This finding was also consistence with other investigators (Poggi *et al*., 1990a, b; Martinez *et al*., 1994) who postulated that 1311^{C-T} is a worldwide polymorphism in the Med$^+$ deficient G6PD gene. However, only three cases have been reported (13.6%) including the single Med$^+$/*BclI*$^-$ chromosome in three different Saudi patients which explained by the fact that those mutations are different in origin from Med$^+$/*BclI*$^+$ or such patients might migrate from other countries as India or Pakistan. Analysis of such

exceptional chromosomes Med^{+}/*BclI*$^{-}$ showed a consistence with previous reports from Italy and Kuwait (Cittadella *et al*., 1997; Samilchuk *et al*., 2003).

G6PD deficiency type A^{-} was second common variant in KSA. This variant, has been reported as genetically heterogeneous that located mainly in Africa, Mediterranean region and the Middle East (Beutler, 1991). The latter is considered a result of gene flow from Africa and was identified as a deficient variant with faster electrophoretic mobility (Betke *et al*., 1967). The A^{-} can be produced by any of deficient mutations (202^{G-A}, 463^{C-G}, 542^{A-T}, 680^{G-T}, 968^{T-C} and 1159^{C-T}) on the chromosomes carrying the second mutation 376^{A-G} (A^{+}), which responsible for the fast enzymatic mobility. These mutations produce different deficient variants when present on chromosome without 376^{A-G} as Asahi 202^{G-A}, Malaga 542^{A-T} and Guadalajara 1159^{C-T} (Calabro *et al*., 1993; Hirono *et al*., 2002). Recent studies demonstrated the enzyme activity is not affected unless the nucleotide 376 mutation is present (Town *et al*., 1992). However, a similar clinical behavior has been reported for G6PD among A^{-} which is determined by any of the three different mutations (202^{G-A}, 680^{G-T} and 968^{T-C}) associated with second 376^{A-G} mutation. Most reported cases of A^{-} were caused by the 202^{G-A} mutation in which Asn was replaced with Asp at position 126 of cDNA. Indeed, ten samples (83.3%) carrying 202^{G-A} mutation were found in this study, which is in consistence with previously published in UAE (75%), Kuwait (72.9%) of G6PD A^{-} mutants (Calabro, *et al*., 1993; Samilchuk *et al*., 2003). However, the 968^{T-C} together with 376^{A-G} was identified only in two cases out of twelve A^{-} patients (16.7%), while the third type of A^{-} (680^{G-T} together with 376^{A-G}) not detected in this screening. Therefore a rare mutation different from 202^{G-A} can underlie A^{-} in the Arabian Peninsula (Samilchuk *et al*., 2003).

The third prevalent type of mutation in KSA is the 143^{T-C} (Aures) mutation that was detected in Saudi G6PD deficient variant (6/49 cases; 12.3%), in which Ileucine was replaced by Threonine at position 481. The Aures mutation was mainly limited to Arabs and it was firstly found in Algeria (7%) of G6PD deficiency, then was later identified in Saudi Arabia (35%), Spain (1.1%), Yemeni, Kuwait (1.4%) and UAE (17%), (Vives *et al*., 1997; Ali *et al*., 2002). Bayourni *et al*. (1996) speculated upon the Arabic origin of the Aures mutation and its spreading with the migration of the Peninsular Arabs to North Africa and Spain at the dawn of Islam. The *Bcl*I haplotypes of the Aures cases reported in this study were *BclI*$^{+}$ which is in agreement with previous study in Algerian and Kuwaitis Aures mutations that is located in chromosomes with the 1311^{C-T} allele.

Other three base substitution mutations found in this study (4/49; 8.2%) were missense mutations 1003^{G-A} (Chatham), 871^{G-A} (Viangchan) and 1360^{C-T} (Union) that showed low frequently (2 cases of the Chatharn (4.1%) and one case for each of Viangchan and Union (2.0%). The Chatham mutation has much wider in ethnic distribution, in contrast to Aures and was firstly identified in an Englishman of Indian descent (Bayourni *et al*., 1996). The frequency of Chatham mutations among G6PD deficiency cases varied in different populations, that was reported in Algeria (1%; Town *et al*., 1992), Filipinos (10.2%; Yang *et al*., 2000), Japan (12.5%), Spain (Vives *et al*., 1997), Italy (Cittadella *et al*., 1997), Brazil (around 0.66%), Oman (10%; Daar *et al*., 1996), Indonesia, Jordan (Karadsheh *et al*., 2005), Iran (27%), Kuwait (7.1%; Samilchuk *et al*., 2003) and Malaysia (2.3%; Yusoff *et al*., 2002) of G6PD deficiency and in most cases it located in the chromosomes had the *BclI*$^{-}$ haplotype (Samilchuk *et al*., 2003). Among 49 different mutations in G6PD genes, two Chatham mutations have been identified in exon 9, that classified as class 3 variant according to WHO classification (Betke *et al*., 1967). It is of interest because its missense mutation located at nucleotide 1003 that is near to G6PD Nara mutation located on the same exon (resulted in deletion of 8 amino acids) that caused severe neonatal hyperbilirubinemia and mild hemolytic anemia. The two KSA cases were carrying Chatham mutation on chromosome held *BclI*$^{-}$ haplotype. However, a single Chatham chromosome from each of Algeria and Oman reported as *BclI*$^{+}$ haplotype (Daar *et al*., 1996). These finding could be interpreted either as a result of recombination or as an independent origin of the Chatham mutation. The Chatham mutation exhibits some clustering in the Middle Eastern populations and could lead to speculate that it is the Middle Eastern origin. In this study, a Saudi boy with Afghanistanian origin was suffering from haemolytic anemia aged 12 years carrying G6PD Viangchan 871^{G-A} associated with 1311 $^{C-T}$silent mutation (*BclI*$^{+}$). This mutations was first discovered in Laotian immigrants in Hawwaii (Beutler, 1994; 1996) and subsequently found to be the most common variant in Laotians (Iwai *et al*., 2001), Thailand (Nuchprayoon *et al*., 2002), Malaysia (Ainoon *et al*., 2003, 2004) and moderate variant in Filipinos (Laosombata *et al*., 2005; 11%), while rarely among the Chinese populations (Yang *et al*., 2000) and was not found in Indonesians (Soemantri *et al*., 1995). The presence of Viangchan variant at relatively high frequency (2.0% of G6PD deficiency cases) in this region reflects the heterogeneity of G6PD in Saudis. The 871^{G-A} had a haplotype *BclI*$^{+}$ in this screening that was not determined in any previous publications. The previous studies concerning the

distribution of Y chromosome haplotype suggested a genetic continuum throughout Southeast Asia and Saudi Arabia that sharing G6PD Viangchan variants with same haplotype chromosome (871^{G-A}/*BclI*$^{+}$) certainly support to the theory of the same genetic origin throughout Southeast Asia. The 1360^{C-T} originally described for variants G6PD Union and G6PD Maewo in the oriental population (Beutler, 1991), that replaced Arg with Cys at position 454 of cDNA G6PD gene. It was detected in only one female case which classified as class 2 (severe deficiency) and suffering from chronic hemolytic anemia (Betke *et al*., 1967). This is first time to find such mutation in Saudi Arabia which might be turned out to be frequent in Saudi population among the Mediterranean-like variants if more samples checked later on.

Sequence analysis revealed a new A-G mutation at position 1342. The 1342^{A-G} mutation results in an (448) Ser to Gly amino acid substitution in the G6PD protein. The biochemical features of this mutation have not been characterized. The nucleotide substitution at 1342^{A-G} and 1360^{C-T} are two different variants reported here in exon 11 of the G6PD gene (Table 1). These substitutions accounts for almost one twenty fifth (4.1%) of the G6PD deficient samples examined. Previous findings showed that Union 1360^{C-T} allele caused a severe G6PD deficiency and classified as class 2 (Betke *et al*., 1967) causing mild to chronic hemolytic anemia as reported here and it is not common among Saudis with G6PD deficiency (Ali *et al*., 2002). This novel mutation (1342^{A-G}) in exon 11 had not been reported in any other ethnic groups and was identified in neonatal with jaundice and severe hemolytic anemia. It creates a new *Hae*III restriction site in which a normal fragment (497 bp) was replaced by two fragments of 307 and 190 bp in the presence of this mutation (Fig. 4). It is interesting to note that both substitutions of the two mutants are very cross to each other (6 amino acid apart), it is rather interesting that both substitutions are located in exon 11 of the G6PD gene and both substitutions result in a replacement of an hydrophilic residue by other amino acids, thus 1342^{A-G} mutation may play same functional role for the activity of the enzyme as union mutation. Beutler (1991) has stated that the more severe form of G6PD deficiency is due to mutations clustering near the putative NADP binding site of G6PD, whereas milder forms of G6PD deficiency are mostly caused by mutations near the amino acid end of the enzyme. Although both 1342 and 1360 substitutions are not in the immediate vicinity of the putative NADP binding domain, their close proximity to this region may alter the kinetics of G6PD in such a way that individuals with either of these two variants would be more susceptible to chemical-induced hemolytic crisis. Actually, the two subjects who suffered from favism were found to carry these types of mutations. In contrast, the mutations giving rise to the A^{+} or the A^{-} variants among African Americans are located far away from the putative NADP binding site toward the amino acid end and mostly classified as class 3 (moderate deficiency). It also should be noted that although the nature of the mutations may be a major determinant of clinical severity in G6PD deficiency, other factors such as nutrition, environment and additional genetic factors may play a role in the pathophysiology of G6PD deficiency. The low frequency of 1342^{A-G} and 1360^{C-T} substitutions among Saudis with G6PD deficiency may explain the apparent difference in the clinical presentations between G6PD-deficient American blacks and G6PD deficient Asians. The new 1342^{A-G} variant was designated as G6PD Haweia.

Recent study have shown that different G6PD biochemical variants share the same DNA mutation and vice versa that the same molecular abnormality may be responsible, for different biochemical phenotypes (Beutler, 1994; Vives *et al*., 1997). Crystallization of G6PD could explain why different molecular defects share similar biochemical phenotypes. However, it remains unexplained why amino acids changes in different domain of the tertiary structure should be responsible for similar biochemical properties (Maeda *et al*., 1992; Mason *et al*., 1994). The three-dimensional data cannot yet explain what happen when other molecular abnormalities are involved (Mason *et al*., 1994).

Frameshift deletion mutations: The modified PCR-SSCP analysis using a mini gel and ethidium bromide staining is quite useful for searching various known and unknown mutations rapidly in a large number of specimens with high sensitivity (Tichaud *et al*., 1992; Hirono *et al*., 1994) and had already been reported from many laboratories (Yap and McGee, 1992; Hirono *et al*., 1994). The conformation change of single-stranded DNA bearing mutations could detect about 90% of the G6PD mutations. This technique can easily identified a frameshift deletions more sensitivity than base substitution as shown in Fig. 1. Combined, the PCR-SSCP method and the subsequent direct sequencing technique are now able to determine all types of mutations.

Although nucleotide deletions or nonsense mutations are common molecular abnormalities that may cause a variety of genetic disorders (Hirono *et al*., 1993 and 2002), they are quite rare in G6PD deficiency cases. The extremely low frequency of amino acid deletions in G6PD deficiency might imply that severe tissue dysfunction usually associated with such drastic structural aberration that is lethal unless the involved

region is functionally insignificant. In this study, a 24 bp deletion in a class 1 variant G6PD gene was identified (G6PD Nara). The existence of two tetranucleotide (CCAC) repeats in the region with the nucleotide deletion strongly suggests that the deletion occurred between the tetranucleotides by mispairing in DNA duplication (Fig. 2), the deleted nucleotides are located in nucleotide 953-976 on cDNA predicting an amino acids deletion of TKGYLDDP at residue 319-326. The deletion mutation of G6PD Nara is one of the most marker sequence alteration found in mutant G6PD genes. The unstable nature of G6PD Nara and the resulting clinical expression of persisting hemolytic anemia seem to be compatible with the marked structural alteration (Hirono *et al*., 1993). Among 49 different mutations in G6PD genes, three G6PD mutations (6.1%) have been identified in exon 9 with a faster gel shift mobility as G6PD Nara (Hirono *et al*., 1993). It is interest because the G6PD Nara mutation is distant from both the putative G6P and $NADP^+$ domains (Hirono *et al*., 1989) and the homology in amino acid sequence from various species is consistently low in this region (Hirono and Miwa, 1993; Beutler, 1994). Deletion of 24 bp mutation reduced e nzyme activity, without any notable kinetic abnormalities (Hirono and Miwa, 1993). These findings suggested that, G6PD Nara is not likely to be associated with the essential function of the enzyme and not cause chronic hemolytic anemia (Hirono *et al*., 1993) in contrast to G6PD Vancouver variant which caused chronic hemolytic anemia (Maeda *et al*., 1992). This discrepancy might be attributed to a difference in functional of the mutated domain (Hirono *et al*., 1993). These results suggest that the biochemical properties of G6PD may be conditioned by the three-dimensional structure of the protein and some genetically determined extragenic factors or post translational modification could play a role in causing the specific behavior of G6PD deficiency.

A single Glycine-174 amino acid deletion of G6PD is located in the region coded by exon 6 of G6PD gene at nucleotide positions 516 to 518. This mutation of G6PD is unique because it is the only known deletion mutation in this region identified that cause chronic hemolytic anemia. Interestingly, both 563^{C-A} (188Ser- Phe) and 3 bp deletion a position 516-518 (-Gly174) mutations are located in exon 6, which is closed to the putative G6P-binding domain ((Hirono *et al*., 1995) and includes a highly conserved amino acid stretch (Hirono *et al*., 1995). This variant enzyme called G6PD Taif. In fact, all variants in exon 6 showed normal or mildly deceased enzyme activities, normal kinetic properties and remarkable clinical manifestations suggest that this region closely relate to the important function of enzyme (MacDonald *et al*., 1991; Hirono *et al*., 1995). Therefore any mutations around this region might classified as class 2 (severe G6PD deficiency cases). Seven biochemically characterized variants with lower substrate affinity have so far been reported that carrying base substitution mutations around the putative G6PD binding site (Laosombata *et al*., 2005) causing hemolytic anemia. Actually, Hirono *et al*. (1995) identified two different G6PD variants with 3 bp frameshift deletion, G6PD Uraysu which is located in the region coded by exon 5 of G6PD gene and G6PD Tsukui that is located in the region coded by exon 6 of the G6PD gene. G6PD Nara and G6PD Uraysu are of special interest because the deletion of 28bp from exon 9 and 3 bp from exon 5 are unique mutations of G6PD deficiency where they are the only known deletions mutations that do not cause chronic hemolytic anemia (Hirono *et al*., 1993, 1995). On the other side our mutation of 3 bp deletion (G6PD Taif) and previously identified G6PD Tsukui are both located on the coded region of exon 6 caused chronic hemolytic anemia (Hirono *et al*., 1995) which might explain the relation between interested mutations located specially on exon 6 near or around G6P binding domain (Hirono *et al*., 1989). Because the latter variants cause chronic hemolytic anemia and the former do not, low substrate affinity of the latter variants might be one of the factors responsible for more severe clinical expression of those variants (Beutler, 1991; Hirono *et al*., 1995, 2002). A novel deletion of 3 nucleotides, the GGG triplet in G6PD gene, was located as two direct tetranucleotide (GGGA) repeats suggests that the deletion occurred by mispairing in DNA duplication similar to G6PD Nara (Fig. 2). This deletion predicts a G6PD protein that lacks a Glycine at position 174 that leads to the expression of an unstable and less active G6PD protein. These altered enzyme properties and the unstable messenger RNA (mRNA) of this variant probably cause the chronic hemolytic anemia in this patient. However, 563^{C-T} creates *Mob*II restriction site, but deletion of GGG at position 516-518 mutation abolished *Bsy*I restriction site (Fig. 3).

In conclusion, the nucleotide substitutions associated with G6PD deficiency in KSA are distinctly different from those associated with G6PD deficiency in other ethnic groups. Most importantly, present findings support the postulate that G6PD deficiency is mainly caused by diverse point mutations and this wide spectrum of point mutations may contribute to the heterogeneity in the pathophysiology of G6PD deficiency disease among Saudis. One unique nucleotide substitutions in the G6PD gene is 1342^{A-G} and one frameshift deletion is three bases (del 448 Gly) associated with G6PD deficiency in patient population (Table 1) were found. These two variants have thus so far only been found in Saudi population and have

not been reported in other ethnic groups. This study clearly indicated that most of mutations are single amino acid replacement of transition types (45/49; 91.88%), transversion mutation is not detected here at all, while frameshift mutations or deletions is considerably few (4/49; 8.12%).

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