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Identification of GP1BA Gene Mutations in Iraqi Patients with Bernard-Soulier Syndrome

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ABSTRACT

Background: Bernard-Soulier syndrome (BSS) is a hereditary bleeding disorder distinguished by macrothrombocytopenia and prolonged bleeding time resulting from quantitative or qualitative defects in the GPIb/IX/V complex. The aim of this project was to detect GP1BA mutations in BSS patients.
Methodology: Twenty BSS patients (11 males and 9 females) and twenty healthy individuals (12 males and 8 females) were included in this study. The selected regions in the GP1BA gene were amplified and directly sequenced.
Results: DNA sequence analysis revealed three mutations in the GP1BA gene, one of which was novel.
Conclusion: BSS, like other autosomal recessive disorders, is prevalent in communities with high incidence of consanguineous marriage, emphasizing the importance of genetic analysis for confirming the diagnosis and identifying carriers were usually asymptomatic.
Keywords: Bernard–Soulier syndrome, GPIb-IX-V, GP1BA.

Introduction

Bernard-Soulier syndrome (BSS) is an autosomal recessive bleeding condition discovered by Bernard and Soulier in 1948, caused by a defect in the platelet complex GPIb/IX/V on a quantitative or qualitative level [1]. It is estimated to occur in 1 per million individuals. However, due to under-recognition and misdiagnosis, the prevalence may be much higher than the estimated [2,3]. The most common symptoms of BSS are epistaxis, ecchymosis, hemorrhage after trauma and heavy menstrual bleeding, hematuria and gastrointestinal bleeding rarely occur. Prolonged bleeding time, macthrombocytroopenia, and low/absence GP1b/V/IX expression approve the diagnosis of BSS [4]. The GPIb-IX-V receptor regulates platelet adhesion to proteins in the subendothelial matrix after an endothelial injury, specifically von Willebrand factor (vWF)[5]. GPIb-IX-V is comprised of four polypeptide chains: GPIbα, GPIbβ, GPIX and GPV. Typically, the absence of one of the GPIb-IX-V subunits greatly reduces the surface expression of the entire receptor, resulting in platelet adhesion defects and increased bleeding susceptibility in BSS patients [6]. The GPIb-IX-V subunits are encoded separately by different genes located at chromosomes 17p12 (*GPIBA*), 22q11.2 (*GPIBB*), 3q21 (*GP9*) and 3q29(*GP5*) [7].Various missense, nonsense, and frame shift mutations were detected in the *GP1BA*, *GP1BB*, and *GP9* genes. Most mutations block the matched association of the complex subunits, this results in very low GPIb/IX/V expression on the platelet surface. In other situations, the receptor is normal or slightly reduced but incapable of attach to vWF[5].

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Methodology

Study groups

This study included twenty patients with BSS (11 males and 9 females) aged between (6-42) year with a history of mild to severe bleeding and twenty healthy individuals (12 males and 8 females) aged between (9-54) year. BSS was diagnosed depending on the clinical presentation and hematological indices.

Blood sampling

Three ml of blood were collected by vein puncture in EDTA anticoagulant tubes from all patients and control group.

DNA isolation

Genomic DNA was extracted from whole frozen blood for molecular studies according to the protocol ReliaPrep™ Blood gDNA Miniprep System.

Amplification of genomic DNA

Polymerase chain reaction (PCR) was used to amplify selected regions of the GP1BA gene using primer pairs previously described by Afrasiabi et al. [8]. PCR primers sequences, positions and the size of each PCR product and PCR components are shown in tables (1,2). Thermocycling conditions were as follows: an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation for 30 sec at 95 °C, annealing for 30 sec at 66 °C , and extension for 30 sec at 72 °C, and a final extension step at 72oC for 7 min. PCR products were then fractionated on 1% agarose gel with a ladder (100 bp) and visualized.

Table 1. Primers sequences and positions

Primer	Seq.	Position	Product (bp)
GpIb_A1-F	5`-GAGAGAAGGACGGAGTCGAG-3`	2761–2780	457
GpIb_A1-R	5`-GGTTGTGTCTTTCGGCAGGT-3`	3218–3199	
GpIb_A2-F	5`-CTGTGAGGTCTCCAAAGTGG-3`	3125–3144	460
GpIb_A2-R	5`-TAGCCAGACTGAGCTTCTCC-3`	3585–3566	
GpIb_A3-F	5`-AAGGCAATGAGCTGAAGACC-3`	3511–3530	597
GpIb_A3-R	5`-CTTGTGTTGGATGCAAGGAG-3`	4108–4087	
GpIb_A4-F	5`-CACAAGCCTGATCACTCCAA-3`	4394–4413	584
GpIb_A4-R	5`-TTCTCTCAAGGTCCCCAAAC-3`	4978–4959	

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Table 2. PCR reaction components

Component	Component
Master Mix (Ready-to-use):TaqDNA, polymerase ,dNTPs, MgCl2	12.5 µl
Forward primer	1 µM
Reverse primer	1 µM
DNA template	2 ng/µl
Nuclease free water	8.5
Final volume	25
Aliquot per single rxn	23 µl of master mix per tube and add 2 µl of template

PCR products sequencing

The PCR products were directly sequenced using an automated sequencer (ABI3730XL Genetic Analyzer; Applied Biosystems) by(Macrogen Corporation – Korea). Geneious software was used for analysis of data and determining sequence variation between samples.

Statistical analysis

The Hardy-Weinberg equilibrium (H-WE) of the detected genotypic distribution was demonstrated using chi-square statistic. WinPepi software was used to determine odds ratio (OR), etiological fraction (EF) and preventive fraction (PF), confidence interval (C・I) of odds ratio at 95%. The differences for all relations were calculated using a two-tailed *P*-value at a significant level less than 0.05.

Results

This study included 20 patients with BSS (11 males and 9 females) aged between (6-42) year. Consanguineous marriages in parents were reported in 60% of patients. The main clinical manifestations were epistaxis, easy bruising, menorrhagia and ecchymosis. Initial laboratory tests revealed variable thrombocytopenia, prolonged bleeding time (BT), and large platelets on peripheral blood smear.

Direct sequencing analysis of twenty BSS patients revealed three mutations in the *GP1BA* gene, two were previously reported and one novel alteration. The first **c.482 C > T** (rs6065) is a missense mutation lead to replacement of nucleotide C to T at position chr17:4933086 (GRCh38.p12) in exon 2 of *GP1BA* gene, leading to substitution of amino acid from threonine to methionine at position161, **c.5263 A>T** mutation (rs81663) is replacement of nucleotide A to T at position chr17:4932557 (GRCh38.p12) in intron 1 of *GP1BA* gene, and **c.6040 T>G** is a novel missense mutation, located at chr17: 4933334 (GRCh38.p12) in exon 2 of *GP1BA* gene lead to substitution of tyrosine to aspartic acid at position 244 of the GPIbα subunit (figure 1).

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c.482C>T was presented with two alleles (*C* and *T*) and three genotypes (CC, CT and TT). These genotypes showed deviation from HWE in BSS patients and control group, because there was significant difference between the expected and the observed frequencies of the genotype ($p \leq 0.05$). Comparing BSS patients to control shown that genotype frequencies of CC (85 % vs. 90%) and CT (10% vs. 5%), while the third genotype (TT) was observed with a frequency 5% in BSS patients and control. The CC,CT and TT genotypes were non-significant difference (p higher than 0.05) in comparison between control and BSS patient in the Fisher's exact test. It was also noticed the frequency of mutant allele (*T*) show non-significant difference in BSS patients compared with control individuals (10 vs. 7.5%; OR = 1.37 ; PF = 0.1;EF= 0.02; 95% C.I. = 0.29 – 6.43), tables(3,4). **c.5263 A>T** was presented with two alleles (*A* and *T*) and three genotypes (AA, AT and TT). These genotypes were in agreement with HWE in BSS patients and controls as there was non- significant difference between the observed and expected frequencies of the genotype. Comparing BSS patients to control revealed that frequencies of AA genotype (90 % vs. 95%) and AT (10% vs. 5%). The frequencies of these genotypes were non-significant difference (p higher than 0.05) in comparison between control and BSS patient in the Fisher's exact test. The TT genotype was not observed in BSS and in control. The frequency of allele (*A*) in BSS patients compared with control individuals was (95 vs. 97.5%), while the frequency of mutant allele (*T*) was (5 vs. 2.5) in BSS patients and control respectively. The frequency of alleles were non-significant difference in comparison between BSS and control (p higher than 0.05),table (3). **c.6040 T>G** was presented with two alleles (*T* and *G*) and three genotypes (TT, TG and GG). These genotypes showed deviation from HWE in BSS patients. The TTgenotype showed a decreased frequency in BSS patients compared to controls (85 *vs*. 100%). The GG show non-significant difference between BSS and healthy subject in the two- tailed Fisher's exact test ($p > 0.05$), the frequency of GG genotype increased in BSS (15% *vs*.0.0%), GG considered as a risk factor in BSS patients(OR=8.2). The heterozygote genotype (TG) was not observed in patients and healthy individuals. The mutant allele (*G*) show a significant difference between BSS patients and control ($p \leq 0.05$) with frequency (15 vs. 0%) and consider risk factor associated with BSS patients (OR=15.2), tables(3,4).

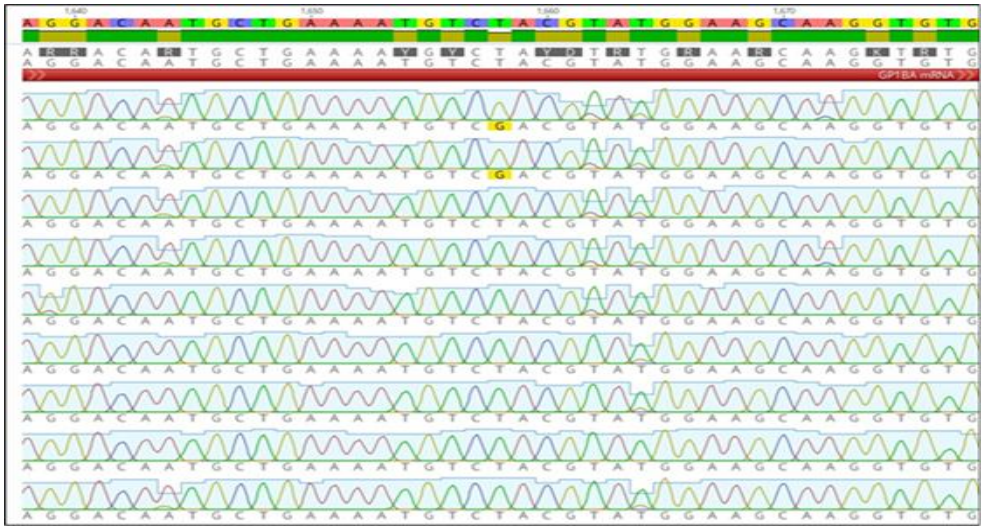

Figure 1. Chromatograms of c.6040 T>G variant in *GP1BA* gene

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Table 3. Genotype/allele frequencies for BSS patients and control

BSS (20)				**Control (20)**		
GP1BA gene SNP	**observed N(%)**	**expected N(%)**	**HWE P value**	**observed N(%)**	**expected N(%)**	**HWE P value**
,c.6040 TAC>GAC						
TT	17(85	72.25)(14.45		20(100)	20	
TG	0 (0)	5.1(25.5)	0.001	0 (0)	0	NS
GG	3(15)	0.45(2.25)		0 (0)	0	
T	34(85)	ND		40(100)	ND	
G	6(15)	ND		0(0)	ND	
,c.5263 A>T, rs81663						
	18(90)	18.05(90.25)		19(95)	19.01(95.05)	
AA	2(10)	1.9 (9.5)		1(5)	0,97(4.85)	
AT	0(0)	0.05(0.25)	NS	0(0)	0.01(0.05)	NS
TT	38(95)	ND		39(97.5)	ND	
A	2(5)	ND		1(2.5)	ND	
T						

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,c.482 C > T, rs6065						
CC	17(85)	16.2(81)		18(90)	17.1(85.5)	
CT	2(10)	3.6(18)	0.05	1(5)	2.77(13.85)	0.005
TT	1(5)	0.2(1)		1(5)	0.11(0.55)	
C	36(90)	ND		37(92.5)	ND	
T	4(10)	ND		3(7.5)	ND	

HWE=Hardy-Weinberg equilibrium, ND=Not determined, N: Number, NS=Non- significant.

Table 4. Epidemiological parameters for BSS patients

GP1BA gene polymorphism	**OR**	**EF**	**PF**	**p-value**	**95% CI**
c.6040 T > G (GG)	8.2	0.14	0.04	0.23	0.43-157
(G)	15	0.14	0.02	0.02	0.86 - 270
c.5263 A>T, rs81663 (AT)	2.11	0.05	0.11	1	0.19-
(T)	2.05	0.02	0.05	1	23.80
					0.18 - 22.8
c.482 C > T, rs6065 (CT)	2.11	0.05	0.11	1	0.19-23.8
(TT)	1	0	0.13	1	0.06-15.9
(T)	1.37	0.02	0.1	1	0.29 - 6.43

OR: Odds ratio, EF: Etiological Fraction, PF: Preventive Fraction, Cl: Confidence interval.

Discussion

Direct sequence analysis revealed that there are three mutations in the *GP1BA* gene.The first **c.482 C > T** (rs6065) is a missense variant in exon 2 of *GP1BA*gene, leading to substitution of

amino acid from polar (threonine) to hydrophobic (methionine) at position161. Thr161Met dimorphism affects the conformation of the N-terminal flanking region and leucine-rich repeats (LRR) of GPIbα. The vWF binding region is found in the LRR (19-281 aa) of the extracellular domain of GPIbα and by this (Thr161Met) influences vWF binding [9]. **c.5263 A>T**(rs81663) is located in intron 1 of *GP1BA* gene. Intronic variant although not changing the predicted protein sequence, but have an important effect on gene expression by several ways, for example can impact alternative splicing by interfering with splice site recognition and can significantly affect gene expression[10]. Intron can increase transcript level by influencing the rate of transcription, transcripts stability and nuclear export. Furthermore, introns can improve the efficiency of mRNA translation [11].

c.6040 T>G is a novel missense variant, located in exon 2 of *GP1BA* gene, lead to substitution of neutral amino acid (tyrosine) to acidic amino acid (aspartic acid) at position 244 of the GPIbα subunit. A nucleotide variation that causes an amino acid replacement can alter the tertiary structure of the GPIb-IX-V complex and possibly change platelet adhesive functions[12]. c.6040 T>G substitution located in the leucine-rich repeat C-terminal region (221-281 aa) of GPIbα subunit[13]. The LRR sequences are necessary for stability of four subunits and suitable configuration of the GPIb-IX-V receptor and involved vWF binding site, therefore mutations of conserved leucine residues are especially deleterious. The variations within the LRR resulted in a more substantial alteration in the formation of GPIbα as well as a significant disturbance in the binding of ligands [14,15].

Conclusion

Genetic analysis revealed a various *GP1BA* gene alterations in BSS patients. Identification of mutations is critical for genetic diagnosis of the affected families in populations such as Iraq where consanguineous marriage is prevalent.

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