Hemoglobin Subunit Beta Gene Polymorphism rs33949930 T>C and Risk of Sickle Cell Disease—A Case Control Study from Tabuk (Northwestern Part of Saudi Arabia)

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Abstract

Background: Sickle cell disease and sickle cell trait are common erythrocyte disorders that are most often caused by a point mutation (rs334, designated HbS) in the hemoglobin beta gene (HBB); however of this fact, there is extreme variability in occurrence and clinical presentation of sickle cell disease which may be explained by some other genetic changes associated with the gene. In the present study we examined the association between HBB gene polymorphism rs33949930 T>C in the occurrence of sickle cell disease in Saudi Arabia population. Materials and Methods: A case control study of 100 sickle cell disease patients and 100 healthy controls from Tabuk, Saudi Arabia. HBB gene rs33949930 T>C polymorphism was analyzed using Allele specific polymerase chain reaction technique. Results: It was observed that the genotype percentages TT, TC and CC among the patients with sickle cell disease were 63.0%, 35.0% and 2.0% and healthy controls were 68.0%, 27.0% and 5.0% respectively. Allele frequency for T allele was observed to be fT = 0.20 and fT = 0.19, whereas for C allele was fC = 0.80 and fC = 0.81 among cases and controls respectively (p = 0.29). Compared to the TT genotype, the odds ratio of 1.4 (95% CI 0.76 - 2.57), risk ratio of 1.2 (95% CI 0.86 - 1.65) and risk difference of 8.4 (-6.66 - 23.38) for heterozygous genotype of HBB rs33949930 T>C was observed in relation to sickle cell disease. In addition, some difference in the laboratory values was observed among sickle cell disease patients with the different variants of HBB gene rs33949930 T>C polymorphism, especially the carriers of heterozygous TC genotype;

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however, the difference doesn’t reach to statically significant number. Conclusion: Present study suggested that there was not any significant association between HBB gene rs33949930 T>C polymorphism and occurrence of sickle cell disease. However, the heterozygous TC genotype of the polymorphism showed some higher ratios among cases as compared to healthy control group.

Keywords
Hemoglobin Subunit Beta (HBB), Sickle Cell Disease (SCD), Tabuk-Northwestern Part of Saudi Arabia

1. Introduction
Sickle cell disease (SCD) is a global public health disorder that affects millions of people across the globe. It is a monogenic disorder caused by an A-to-T point mutation at the sixth codon of the hemoglobin beta gene on chromosome 11p15.5. The mutation leads the substitution of a valine amino acid for a glutamic acid that produces abnormal hemoglobin S (Hb S), which polymerizes in the deoxygenated state, resulting in physical deformation or sickling of erythrocytes. Globally sickle cell disease is the most common genetic disorder [1] with highest prevalence in Middle East, Mediterranean regions, Southeast Asia, and sub-Saharan Africa especially Nigeria [2]. As per the World Health Organization published global prevalence map of sickle cell disease, about 20 - 25 million individuals globally suffer from homozygous sickle cell disease [3]. SCD was first reported by Lehmann et al. in the eastern province of Saudi Arabia [4] and latter studies have reported that SCD is the most common genetic disorder in Saudi Arabia with the highest frequency in the eastern and southwestern province of Saudi Arabia [4]-[8]. Sickle cell disease carriers in Saudi Arabia ranged from 2% to 27%, and in some areas up to 1.4% had sickle cell disease [4]-[8]. In addition to environmental factors it has been observed that various genetic determinants may be involved in the risk of developing sickle cell disease [9].

Rapid advances made in understanding the molecular genetics of SCD in the early part of the 20th century have not been matched by comparable progress towards understanding its clinical complications, and developing effective therapies. Many studies have investigated the effect of genetic variants in the BCL11A, the HMIP (HBS1L-MYB intergenic polymorphism) locus, in addition to the HBB locus, which is known to be associated with fetal hemoglobin (HbF) levels, a major modulator of the disease phenotype. Genotyping was performed for the BCL11A rs11886868 and rs34211119; HMIP rs9399137, rs189600565, rs7776196, rs34778774, and rs53293029; HBG2 Xmn1 polymorphism rs7482144; and −68C>T HBD promoter polymorphism. All the 3 quantitative trait loci were associated with HbF levels in Indian patients with SCD [10]-[13].

The highest difference was seen in the Xmn1 single-nucleotide polymorphism, which accounted for 11% of the trait variance, the BCL11A rs11886868 for 3.65%, whereas the HMIP rs9399137 for 3.8%. Several studies indicated that the BCL11A, HMIP, and β-globin regions were associated with increased HbF levels in different populations; and investigation of these genotypes with respect to pain crisis is warranted in different population, which may help in prognostication, as also a genome-wide association study, which may help uncover new loci controlling HbF levels [14] [15].

Multiple SNP variants in these gene regions are associated with higher levels of FH and a milder course of disease. Together with the SNPs in the γ-globin region of the β-globin cluster, these loci account for more than 20% of the variance in FH levels among SCA patients in the United States and Brazil. SCD is prevalent in Saudi Arabia and is probably underestimated. The variable genetic origin and variable clinical phenotype of SCD between the East and West parts of Saudi Arabia make it possible to further pursue research on genetic, clinical, and environmental modifiers of SCD [16] [17].

Hemoglobin subunit beta gene polymorphism rs33949930 T>C may be associated with sickle cell diseases. In this polymorphism of Hemoglobin Subunit beta 1, the amino acid valine is replaced by acetylanaline or thiamine to cytosine. Kamel et al. [18] investigated that a Qatari family with an electrophoretically fast-moving hemoglobin that they found contained an abnormal beta chain with the sequence met-glu-his-leu at the NH2-end. Substitution of glutamic acid for valine at beta 1 apparently prevented removal of the initiator methionine. The methionine was blocked by a molecule not completely identified. No clinical consequences were observed in heterozygotes. This variant was numbered based on the first amino acid of the mature protein. In the gene-based
system of counting, this variant is VAL2GLU.

There is a need for systematic, prospective studies that document the prevalence, molecular and clinical epidemiology of SCD in different areas of Saudi Arabia to help predict disease severity, risk stratify patients to receive early intensive care or continued symptomatic care, and describe the problems currently faced by patients affected with SCD in Saudi Arabia. In order to understand the biological basis of various diseases especially the genetic disorders, single nucleotide polymorphisms (SNPs) are being intensively studied with promising conclusions.

In the present study we examined the association between HBB gene polymorphism rs33949930 T>C in the occurrence of sickle cell disease in Saudi Arabia population. To the best of our knowledge, no information is available concerning the association between HBB gene polymorphism rs33949930 T>C and sickle cell disease.

2. Material and Methods

The study was conducted in the Division of Cancer Molecular Genetics, Prince Fahd Bin Sultan Research chair, University of Tabuk, Saudi Arabia. The study was approved by the ethics Committee, University of Tabuk. The samples were obtained from the hospital stored at −30˚C and were collected from the patients visiting hospital for routine checkup.

Study population: The study included 100 clinically confirmed Sickle cell disease cases and 100 healthy controls. The samples were also collected from the healthy controls visiting Hospital for routine checkup. The description of the laboratory characteristics for patients with sickle cell disease are summarized in Table 1.

2.1. Genotype Analysis

Peripheral blood sample (3 - 5 mL) from each participant was drawn into an EDTA vial and genomic DNA was extracted by following manufacturer’s protocol of DNA extraction kit (Qiagen). ASO-PCR was performed to determine the (rs33949930) T>C HBB gene among sickle cell disease patients and compared with healthy controls. PCR was performed in a final volume of 25 μL containing 5 μL of 50 ng genomic DNA, 12.5 μL of PCR-master mix (Promaga), 0.25 μL of 25 pmol/L of each primer (Table 2) and remaining nuclease free ddH2O. PCR program was started with an initial denaturation at 95˚C for 5 minutes, followed by 40 cycles of denaturation at 95˚C for 30 s, annealing at 60˚C for 45 s, extension at 72˚C for 45 s, and completed with a final elongation step at 72˚C for 5 minutes. Negative control with ddH2O instead of DNA template was included in each PCR run. A blind case/control analysis was performed with approximately more than 10% random samples were selected for confirmation and the results were 100% concordant. PCR products were visualized on ethidium bromide stained 2% agarose gel (Figure 1).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Mean ± SD</th>
<th>Range (min - max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb</td>
<td>13.97 ± 1.87</td>
<td>12.70 (5.50 - 18.20)</td>
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<tr>
<td>WBC</td>
<td>7.17 ± 1.66</td>
<td>13.31 (3.69 - 17.00)</td>
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<tr>
<td>RBC</td>
<td>5.11 ± 0.74</td>
<td>3.72 (3.40 - 7.12)</td>
</tr>
<tr>
<td>HCT</td>
<td>39.95 ± 5.08</td>
<td>34.80 (18.00 - 52.8)</td>
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<tr>
<td>MCV</td>
<td>79.91 ± 6.06</td>
<td>27.00 (65.00 - 92.00)</td>
</tr>
<tr>
<td>RDW</td>
<td>12.7090 ± 1.22</td>
<td>7.00 (11.00 - 18.00)</td>
</tr>
<tr>
<td>PLT</td>
<td>303.90 ± 66.86</td>
<td>266.00 (190.00 - 456.00)</td>
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<tr>
<td>HbA1</td>
<td>63.44 ± 13.44</td>
<td>94.50 (3.10 - 97.60)</td>
</tr>
<tr>
<td>HbA2</td>
<td>4.06 ± 3.85</td>
<td>23.90 (2.30 - 26.20)</td>
</tr>
<tr>
<td>HbF</td>
<td>0.7680 ± 1.75</td>
<td>14.80 (0.00 - 14.80)</td>
</tr>
<tr>
<td>HbS</td>
<td>36.40 ± 7.44</td>
<td>57.40 (25.00 - 82.40)</td>
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Table 2. Primer sequences for HBB rs33949930 (T>C) polymorphism.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>AT</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1 (Forward primer 1)</td>
<td>5’-ACGGCAGACTTCTCCTCAGGAGTCAGATGCAC-3’</td>
<td>63°C</td>
<td>282 bp</td>
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<tr>
<td>F2 (Forward primer 2)</td>
<td>5’-ACGGCAGACTTCTCCTCAGGAGTCAGATGCAT-3’</td>
<td>63°C</td>
<td>282 bp</td>
</tr>
<tr>
<td>R (Common reverse)</td>
<td>5’-TATCTTGAAGGGAGGCCGCTGAGGTTT-3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SCD patient - P1 → Positive for TT genotype and negative for CC genotype - homozygous for TT allele
SCD patient - P2 → Positive for both genotypes TT and CC - heterozygous
SCD patient - P3 → Negative for TT genotype and positive for CC genotype - homozygous for CC allele
SCD patient - P4 → Positive for TT genotype and negative for CC genotype - homozygous for TT allele

Figure 1. Agarose gel electrophoresis of PCR amplification of hemoglobin subunit beta gene polymorphism rs33949930 T>C in sickle cell disease.

2.2. Statistical Analysis

Statistical analysis was performed using SPSS 16.0 software. Assessment of the correlations between genetic carrier status and HBB gene polymorphism was carried out using the Chi-Square or Fisher Exact test. HBB gene variants and risk of sickle cell disease were estimated by computing the odds ratios (OR), risk ratio (RR) and risk difference (RD) with 95% confidence intervals (CIs) from multivariate logistic regression analysis. Student t test was used to calculate Mean ± SD and allele frequencies among cases as well as controls were evaluated by using Hardy-Weinberg equilibrium test. A p value < 0.05 was considered significant.

3. Results

3.1. Case-Control Genotype Distribution

In the present study while analyzed the HBB gene rs33949930 (T>C) polymorphism (70599 T>C) polymorphism, it was observed the genotype percentages TT, TC and CC among the patients with sickle cell disease were 63.0%, 35.0% and 2.0% and healthy controls were 68.0%, 27.0% and 5.0% respectively. While calculating the allele frequency, a slight non statistically significant difference (p = 0.29) was observed with T allele frequency of fT = 0.20 and C allele frequency of fC = 0.80 among cases and T allele frequency of fT = 0.19 and C allele frequency of fC = 0.81 among healthy controls (Table 3).

3.2. HBB Gene rs33949930 (T>C) Polymorphism and Risk of Sickle Cell Disease

Logistic regression was used to estimate associations between the genotypes and risk of SCD (Table 4). Compared to the TT genotype, the odds ratio of 1.4 (95% CI 0.76 - 2.57), risk ratio of 1.2 (95% CI 0.86 - 1.65) and risk difference of 8.4 (~6.66 - 23.38) for heterozygous genotype of HBB (70599TC) was observed in relation to sickle cell disease among population of Saudi Arabia.

3.3. HBB Gene rs33949930 (T>C) Genotypes and Laboratory Characteristics

Upon correlating the laboratory findings of sickle cell disease patients with the different variants of HBB gene
rs33949930 (T>C) polymorphism, it was observed that there was some difference among the values but the different doesn’t reach to statically significant number (Table 5). In relation to rs33949930 (T>C) genotypes (TT, TC and CC) the mean HbA1 and HbA2 levels were 63.26, 63.80, 62.50 and 4.1, 4.03, 2.90 respectively. While comparing the HbF level with the HBB rs33949930 (T>C) genotypes CC homozygous carriers showed lower level of HbF compared to patients with TT homozygous and TC heterozygous genotypes; however HbS level was almost similar in association with different variants of rs33949930 (T>C) polymorphism (Table 5).

4. Discussion

The HBB gene in humans located on chromosome 11p15.5 encodes for a protein called beta-globin, which is an important subunit of hemoglobin. In adults, hemoglobin normally consists of four protein subunits: two subunits of beta-globin and two subunits of another protein called alpha-globin, encoded by HBA gene. Among these subunits several changes at nucleotide level has been observed and has been found to be associated with various genetic disorders like sickle cell disease.

Homozygosity for a single β-globin gene mutation (β6GAG → GTG) has been found to be the main cause of sickle cell disease, however of this fact, there is extreme variability in occurrence and clinical presentation from

<table>
<thead>
<tr>
<th>Genotype</th>
<th>TT (T allele frequency)</th>
<th>TC (C allele frequency)</th>
<th>p value</th>
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<tbody>
<tr>
<td>Cases (n = 100)</td>
<td>63 (63.00)</td>
<td>35 (35.00)</td>
<td>2 (2.00)</td>
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<tr>
<td>Controls (n = 100)</td>
<td>68 (68.00)</td>
<td>27 (27.00)</td>
<td>5 (5.00)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Control (n = 100)</th>
<th>Cases (n = 100)</th>
<th>OR (95% CI)</th>
<th>RR (95% CI)</th>
<th>RD (95% CI)</th>
<th>p value</th>
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</thead>
<tbody>
<tr>
<td>TT (reference)</td>
<td>68</td>
<td>63</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>5</td>
<td>2</td>
<td>0.43</td>
<td>(0.08 - 2.31)</td>
<td>0.73</td>
<td>(0.44 - 1.19)</td>
</tr>
<tr>
<td>TC</td>
<td>27</td>
<td>35</td>
<td>1.4</td>
<td>(0.76 - 2.57)</td>
<td>1.2</td>
<td>(0.86 - 1.65)</td>
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</tbody>
</table>

<table>
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<tr>
<th>Variables</th>
<th>Mean ± SD</th>
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<tr>
<td>Hb</td>
<td>13.78 ± 1.94</td>
<td>14.33 ± 1.76</td>
</tr>
<tr>
<td>WBC</td>
<td>5.04 ± 1.78</td>
<td>7.02 ± 1.45</td>
</tr>
<tr>
<td>RBC</td>
<td>5.01 ± 0.72</td>
<td>5.29 ± 0.76</td>
</tr>
<tr>
<td>HCT</td>
<td>39.31 ± 5.09</td>
<td>41.02 ± 5.04</td>
</tr>
<tr>
<td>MCV</td>
<td>79.94 ± 6.18</td>
<td>79.65 ± 6.02</td>
</tr>
<tr>
<td>RDW</td>
<td>12.74 ± 1.29</td>
<td>12.65 ± 1.10</td>
</tr>
<tr>
<td>PLT</td>
<td>3.00 ± 60.47</td>
<td>3.10 ± 75.33</td>
</tr>
<tr>
<td>HbA1</td>
<td>63.26 ± 14.96</td>
<td>63.80 ± 10.75</td>
</tr>
<tr>
<td>HbA2</td>
<td>4.11 ± 3.91</td>
<td>4.03 ± 3.89</td>
</tr>
<tr>
<td>HbF</td>
<td>0.99 ± 2.17</td>
<td>0.38 ± 0.21</td>
</tr>
<tr>
<td>HbS</td>
<td>36.61 ± 8.95</td>
<td>35.99 ± 3.82</td>
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asymptomatic to a very severe of the patients with sickle cell disease [19] [20]. The reason for this variability
may be explained by some other genetic changes, especially the SNPs in other positions of the HBB gene. In the
present study we examined the association between HBB gene polymorphism rs33949930 (T>C) in the develop-
ment of sickle cell disease in Saudi Arabia population.

Structural analysis of a fast-moving hemoglobin variant, present in three members of a Qatari family, identi-
fied a Val → Glu substitution at position 1 (NA1) of the β-chain. The introduction of this glutamic acid residue
prevents the removal of the initiator methionine, thus extending the N-terminus by one residue to Met-Glu-His-
Leu-Thr. The methionine residue is blocked by an as yet not completely identified molecule. The presence of the
variant in a heterozygote does not have clinical consequences [21]. Four hemoglobin variants had previously
been described that involve the first codon of the HBB gene: Hb Doha (141900.0069), Hb South Florida
(141900.0266), Hb Niigata (141900.0471), and Hb Raleigh (141900.0233). Although none of these variants
cause any significant clinical problems, mutations of the first codon are of interest because of their potential in-
terference with cotranslational modification at this site during beta-globin synthesis. In eukaryotes, the trans-
lation of all peptide mRNAs starts at an AUG codon, producing methionine at the beginning of the nascent pe-
tide chain. Fisher et al. [22] identified a new Hb variant, Hb Watford, in which a GTG-to-GGG substitution
caused a change of the first amino acid of the beta-globin chain from methionine to glycine, mimicking the
gamma-globin chain. The proband was a 48-year-old female of Jewish extraction who was evaluated for chronic
mild anemia. Another mutation was found in cis with the val → gly mutation: Cap+36G-A.

In the present study we observed the similar distribution of the genotype percentages among cases and con-
trols; accept slight difference in the heterozygous TC genotype of HBB gene polymorphism rs33949930 (T>C),
which was higher among cases than controls. Allele frequency evaluation revealed non-significant distribution
among the study groups. Odds and risk ratios were higher for heterozygous TC genotype with respect to the
normal homozygous TT genotype of HBB gene polymorphism rs33949930 (T>C) among population of Saudi
Arabia. When we correlated the laboratory findings of the sickle cell disease patients with the different variants
of HBB gene rs33949930 (T>C) polymorphism observed some differential values; especially in HbA2 and Hbf
levels which were lower among patients carrying the heterozygous TC genotype; however Hbs level was almost
similar in association with different variants of HBB rs33949930 (T>C) polymorphism.

5. Conclusion

Our data suggest that there was not any significant association between HBB gene rs33949930 (T>C) polymor-
phism and occurrence of sickle cell disease. However, the heterozygous TC genotype of the polymorphism
showed some higher ratios among cases as compared to healthy control group. The findings of the present study
are limited due to smaller sample size under study groups; the importance of the heterozygous TC genotype of
HBB gene rs33949930 (T>C) polymorphism in sickle cell disease can be validated by large sample size studies.

Acknowledgements

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Competing Interests

The authors declare that they have no competing interests.

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