Serum Level of High-Mobility Group Box Protein 1 as a Potential Treatment Target in Egyptian Sickle Cell Disease Patients

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Abstract

BACKGROUND: During tissue injury, high mobility group box 1 (HMGB1) is passively released from necrotic cells and actively secreted by inflammatory cells. Extracellular HMGB1 acts as an amplifier of toll-like receptor-dependent inflammation. HMGB1 serum level was reported in the literature to be a marker of severity and a significant contributor to the progression of the inflammatory process in different diseases. Sickle cell disease (SCD) is no longer considered a monogenic disease but rather considered an inflammatory condition with augmentation of sterile inflammation during vaso-occlusive crises (VOCs). The treatment modalities expanded, given this knowledge to include P-selectin monoclonal antibody (Crizanlizumab).

AIM: Other inflammatory markers are being evaluated as a target for treatment.

METHODS: We studied HMGB1 quantitative trait locus reference sequence rs2249825 in peripheral blood samples using real-time polymerase chain reaction (RT-PCR) in the thermocycler (StepOne™ Real-Time PCR System) and its serum level using a two-site enzyme-linked immunosorbent technique in both SCD patients and healthy controls. SCD patients were further subclassified into different genetic subgroups (Hb SS, Hb Sβ+, or Hb Sβ+β0). The salient increase in HMGB1 serum level, both in the steady-state and in vaso-occlusion of SCD, suggests it as a potential additive therapeutic target in SCD in general and in vaso-occlusions in specific.

RESULTS: Both the SCD patients and the control group had comparable HMGB1 genotype frequencies (p > 0.05) using different genetic models. SCD patients at their steady-state showed statistically significantly higher serum HMGB1 levels than the healthy controls, a median of 0.6 ng/ml with a range of 0.1–85 ng/ml versus a median of 0.2 ng/ml and a range 0.2–7.4 ng/ml (p < 0.0001), respectively. Statistically significant skewed high serum HMGB1 levels than the healthy controls, a median of 0.6 ng/ml with a range of 0.1–85 ng/ml versus a median of 0.2 ng/ml and a range 0.2–7.4 ng/ml (p < 0.0001), respectively. Statistically significant skewed high serum HMGB1 in the VOC samples in contrast to the steady-state samples was observed in the SCD patients with a median of 3.2 ng/ml and a range of 0.3–76.4 ng/ml versus a median of 0.2 ng/ml and a range 0.2–7.4 ng/ml (p < 0.0001), respectively.

CONCLUSION: The salient increase in HMGB1 serum level, both in the steady-state and in vaso-occlusion of SCD, suggests it as a potential additive therapeutic target in SCD in general and in vaso-occlusions in specific.

Introduction

In sickle cell disease (SCD), hemoglobin S is produced when valine replaces glutamic acid in the sixth amino acid of the β globin chain of the hemoglobin molecule due to homozygous point mutation (GAG > GTG) in the sixth codon of the beta (β) globin gene [1, 2]. At low oxygen levels, the abnormal hemoglobin polymerizes, making the red blood cells (RBCs) more rigid and deformed, leading to obstruction of the vessels [3]. Furthermore, hemolysis occurs both intra- and extravascular and releases free hemoglobin, which has inflammatory and oxidant effects, leading to endothelial dysfunction. Moreover, other products such as heme, reactive oxygen species (ROS), and reactive nitrogen species (RNS) are released into the circulation together with low nitric oxide (NO), stimulating the release of pro-inflammatory cytokines and growth factors [4, 5]. The pro-inflammatory cytokines are produced by several cells and contribute to the pain that counters the effect of analgesics. Neutrophils, endothelial cells, monocytes/macrophages, fibroblasts, and T-cells are the main cells responsible for the release of interleukins such as IL-12, IL-2, IL-4, IL-5, IL-6, IL-10, IL-8, IL-23, TNF-α, and IFN-γ [6, 7].

Other than the vaso-occlusive crises (VOCs), SCD is usually a multisystemic disorder and presents with a wide spectrum of sequelae, including anemias, mostly of the hemolytic type [8], neurological [9], pulmonary [10], renal [11], musculoskeletal/dermatological [12], hepatobiliary [13], and splenic complications [14]. There is also increased susceptibility to infections with Streptococcus pneumoniae and Haemophilus influenza as a result of splenic atrophy [15].

Among the most frequent presentations of VOC are painful crises occurring in the musculoskeletal system, abdominal pain, and priapism, usually requiring
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β. [23] investigated HMGB1


those who were not receiving HU (3) Absence of blood concomitant Hydroxyurea (HU) capsules as well as inclusion in the study (2) Patients who were receiving acute splenic sequestration) at least one month before episodes/crises (infection, VOC, ACS, stroke, priapism, defined by all of the following: (1) Absence of acute provided consent. Steady-state sickle cell patients were acting as volunteer blood donors and attendees of the pediatric cosmetic outpatient clinics for the management of bat ears. Each participant and/or their guardian provided consent. Steady-state sickle cell patients were defined by all of the following: (1) Absence of acute episodes/crises (infection, VOC, ACS, stroke, priapism, acute splenic sequestration) at least one month before inclusion in the study (2) Patients who were receiving concomitant Hydroxyurea (HU) capsules as well as those who were not receiving HU (3) Absence of blood transfusions in the previous three weeks. Exclusion criteria included those who received a blood transfusion in the last month before sampling.

The SCD patients were genetically subclassified into Hb SS, Hb Sβ, Hb Sβ, and Hb SO Arab based on their hemoglobin separation techniques (HPLC and alkaline electrophoresis) results. The study included a paired VOC sample for 26 patients of the 100 patients. Sickle cell-related pain crises were defined according to the Cooperative Study of SCD as "acute episodes of pain in the abdomen, back, extremities, chest, or head, with no medically determined cause other than a VOC event, that might have had resulted in a medical facility visit and treatment with oral or parenteral narcotic agents, or with a parenteral non-steroidal anti-inflammatory drug." Pain episodes within 14 days were treated as a single episode [24]. The steady-state laboratory parameters were based on measurements obtained during a median of two visits without a change in the HU dose. The annual rate of sickle cell-related VOC crises and disease SCD severity classes were calculated according to Shah et al. [17], with class III being the most severe.

Candidates, both patients and controls, were subjected to clinical assessment, radiological investigations, basic laboratory investigation, The HMGB1 rs2249825 genotyping as well as the measurement of HMGB1 serum level. A volume of 6 ml of venous blood was withdrawn under complete aseptic conditions and distributed as follows:(1) In a sterile vacutainer containing K3-EDTA, 2 ml of blood were delivered to perform the routine automated hemogram. This included Hb estimation, RBCs count, hematocrit value, mean corpuscular hemoglobin, platelet count, and total and differential leucocytic count. These parameters were obtained electronically using Sysmex XS-800i. Furthermore, the examination of Leishman-stained peripheral blood smears for verification of differential leukocyte count was done. Reticulocyte count and ESR measurement were determined. Finally, both alkaline Hb electrophoresis and HPLC were done (2) In a sterile vacutainer containing K3-EDTA, 2 ml of blood were delivered to and stored at –20°C to be used for the genotyping technique. (3) In a sterile serum vacutainer, 2 ml of blood were delivered and immediately centrifuged at 2000-3000 round per minute (RPM) for approximately 20 minutes, to perform high sensitivity CRP, liver function test (LFT), kidney function test (KFT), biochemical hemolytic profile (LDH, total and direct bilirubin) and then stored at –20°C to be used for HMGB1 protein quantification.

High-mobility group box 1 (rs2249825) genotyping

The data from the GTEx database were used to identify correlations between SNPs and

Materials and Methods

The study included 100 steady-state SCD patients and 100 age- and sex-matched control subjects, acting as volunteer blood donors and attendees of the pediatric cosmetic outpatient clinics for the management of bat ears. Each participant and/or their guardian provided consent. Steady-state sickle cell patients were defined by all of the following: (1) Absence of acute episodes/crises (infection, VOC, ACS, stroke, priapism, acute splenic sequestration) at least one month before inclusion in the study (2) Patients who were receiving concomitant Hydroxyurea (HU) capsules as well as those who were not receiving HU (3) Absence of blood
levels of HMGB1 expression. An investigation into the expression of quantitative trait loci (eQTLs) was conducted to determine the functional role of phenotype-associated SNPs, which revealed rs2249825 (3814G/C; genomic number 31,037,903) near the exon as a down regulator of the gene. The dominant allele was G, while the recessive allele was C. DNA was extracted according to the instruction of the ready-made extraction Kit (QiAamp DNA Mini Kit), Catalog number: 51304, QiAGEN (Hulsterweg 82 5912 PL Venlo, The Netherlands). Samples’ quality (quantity of DNA and purity) was assessed using a NanoDrop Q5000 UV-Vis Spectrophotometer. The measurement of DNA quantity was determined by measuring the absorbance (A) at 260 nanometer (nm), and at 320 nm, multiplying by the dilution factor, and using the relationship that an A\text{260} of 1.0=50\mu g/ml pure dsDNA.

DNA concentration (\mu g/ml) = (A\text{260} reading − A\text{230} reading) × dilution factor × 50\mu g/ml. The total DNA yield is obtained by multiplying the DNA concentration by the final total purified sample volume. DNA yield (\mu g) = DNA concentration × total sample volume (ml). The measurement of DNA purity was determined by the optical density (OD) at 260 and 280nm. DNA purity (A_{\text{260}}/A_{\text{280}}) = (A_{\text{260}} reading − A_{\text{230}} reading) ÷ (A_{\text{280}} reading). Good-quality DNA was considered to have an A_{\text{260}}/A_{\text{280}} ratio of 1.7–1.9 and A_{\text{260}}/A_{\text{230}} greater than 1.5.

The 20 \mu l total volume of each component needed for the assay was calculated as follows: (1)10\mu l TaqMan Universal polymerase chain reaction (PCR) Master Mix. (2) 1\mu l HMGB1 TaqMan SNP Genotyping Assay (20x). (3) 4 \mu l DNAse free water (adjusted according to the DNA concentration of each sample). (4) DNA template. The rs2249825 genotype was assayed by RT-PCR using a thermocycler (StepOne™ Real-Time PCR System, Catalog number: 4376357) to amplify the specific DNA fragment of the HMGB1 gene using TaqMan™ SNP Genotyping Assay. (Catalog number: 4351379, Applied Biosystems: Campus (Foster City, California). The PCR amplification program used was an initial denaturation at 95°C for 10 min, followed by 40 cycles of 92°C for 15 s, followed by a final annealing/extension step at 60°C for 60 s. The PCR-amplified product was detected using the VIC-dye fluorescence (G variant) and the FAM-dye fluorescence (C variant). The presence of both alleles together indicated a heterozygous genotype carrying both the dominant (G) allele and the recessive (C) allele. Different genetic models were constructed with the GC genotype being appreciated as a separate entity in the additive model, added to the GG genotype in the dominant model, or added to the CC genotype in the recessive model. Allelic discrimination plot showing the sickle cell patients’ distribution regarding the HMGB1

genotype, an additive model was created using the thermal cycler [Figure 1].

![Allelic Discrimination Plot](image)

**Figure 1:** Allelic discrimination plot showing the whole sickle cell patients’ distribution regarding the high mobility group box 1 genotype, additive model

**High mobility group box 1 serum level**

Measurement of HMGB1 serum level, both the steady-state and the VOC samples, was done by sandwich enzyme-linked immunosorbent technique (ELISA) (Catalog number: E1635Hu, China), then absorbance at 450 nm was measured using an ELISA reader (R and D Systems, Inc, Minneapolis, MN).

**Statistical analysis of data**

The required sample size for the VOC/steady-state paired samples was calculated utilizing the data obtained by Xu et al. [23]. The paired sample part of the study was planned. The calculation revealed a minimum sample size of 16 (number of pairs) to achieve a power of 95% at an alpha level of 0.05 (two-sided) for detecting a serum HMGB1 mean of the differences of 7 ng/ml between pairs, assuming the standard deviation of the differences to be 7. The data were coded and analyzed using SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 21. Chi-square (v2) test was utilized to validate the true genetic nature of controls by evaluating the deviation from Hardy–Weinberg equilibrium (HWE). Qualitative data were depicted as frequency and percentage. Parametric numerical data were expressed as mean, standard deviation, and range, while nonparametric data were expressed as median and range. For analyzing the difference between frequencies of variables, the Chi-square test was selected. The Kruskal–Wallis and Mann–Whitney tests were conducted to compare nonparametric numerical data. P-values less than 0.05 were considered statistically significant. For Kruskal-Wallis statistically significant results, a post hoc test was employed. For a comparison of paired measurements within each patient, the non-parametric Wilcoxon signed-rank test was used. Bar charts were used for the additive model visualization across the genetic
subtypes of SCD as well as different classes of SCD. For the plotting of paired data, an online gg plot creator (available at https://www.graphpad.com) was utilized. Correlations between quantitative variables were done using the Spearman correlation coefficient.

Results

Patients’ median age was 11 years with a range of 1.7 – 36. There were 58 males and 42 females. Concerning the high prevalence of the SCD in Egyptian subjects, the history of current study patients revealed the majority were a result of consanguineous marriage (61 % of cases), with 51 % of cases having an affected sibling. Concerning the clinical examination of the SCD group, the majority had hepatomegaly and splenomegaly (71% and 64 % of cases, respectively) and did not have calcuric disease (80 % of cases). Regarding the complication experienced by SCD patients, all of the patients and their guardians failed to report transient ischemic attacks, none experienced growth retardation, osteomyelitis, or anemic heart failure. As regards the treatment modalities used by SCD patients, the majority used HU and chelation (84% and 81 % of cases, respectively). During the time of data collection, SCD patients were on a range of 13–25mg/kg (median 18.6 mg/kg/d) of HU.

High mobility group box 1 rs2249825 genotyping

Regarding the analysis of the HMGB1 rs2249825 genotypes distribution, the genotypic distribution of the studied controls was in accordance with the Hardy–Weinberg equilibrium (p > 0.05). Using different genetic models (additive, dominant, recessive, and allele model) in SCD whole cases [Figure 1 and Table 1], within the SCD genetic subtypes (Hb SS, Hb Sβ+) [Figure 2 and Table 1] and the SCD Shah et al. [17] severity classes [Figure 2 and Table 2], no statistically significant difference from the controls was detected.

High mobility group box 1 steady-state serum level

The HMGB1 serum level in the healthy controls compared to steady-state SCD whole study patients was statistically significantly higher value (p < 0.001). Similarly, the HMGB1 serum level was higher in the steady-state Hb SS subjects compared to controls (p = 0.043) [Figure 3 and Table 1]. Further characterization of the SCD group revealed class III severe cases to show higher serum HMGB1 levels than controls, class I and class II (p < 0.0001, p = 0.023, p = 0.015, respectively), [Figure 3 and Table 2].

A statistically significant association between higher serum levels of HMGB1 and thrombotic events and seizure was detected (p = 0.049, p = 0.03, respectively). There was no statistically significant correlation between the HMGB1 rs2249825 genotype and any of the studied clinical parameters (data not shown).

High mobility group box 1 vaso-occlusive crises serum level

The 26 patients with paired samples resulted in a 98.5 % power of the study for detection of a difference between HMGB1 steady-state and VOC.

A statistically significant association was found between the serum level of HMGB1 in the VOC samples and the length of the hospital visit, the greater the HMGB1 serum level, the longer the length of the hospital stay (p < 0.001). In contrast, the study did not reveal a statistically significant correlation between the serum level of HMGB1 in the VOC samples and any of the laboratory parameters (free plasma hemoglobin and serum haptoglobin) known to affect the HMGB1 serum levels, p = 0.699 and p = 0.033 (r = 0.419), respectively. A comparison of paired steady-state and VOC crisis samples revealed the serum HMGB1 was further increased during the crisis (VOC state with a statistically significant difference from the controls was detected.

Table 1: The distribution of high mobility group box protein 1 reference sequence 2249825 genotype and serum level of high mobility group box protein 1 in controls and sickle cell disease subjects of different genetic types

<table>
<thead>
<tr>
<th>HMGB1 rs2249825 genotype</th>
<th>Normal control (n = 100, n (%))</th>
<th>Whole cases (n = 100), n (%)</th>
<th>Hb SS (n = 52), n (%)</th>
<th>Hb Sβ+ (n = 31), n (%)</th>
<th>Hb SS (n = 16), n (%)</th>
<th>Hb Sβ+ (n = 31), n (%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Additive model</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>5 (5)</td>
<td>3 (3)</td>
<td>3 (5.8)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.2</td>
</tr>
<tr>
<td>GC</td>
<td>25 (25)</td>
<td>18 (18)</td>
<td>13 (25)</td>
<td>-</td>
<td>5 (16.1)</td>
<td>26 (83.9)</td>
<td>1 (100)</td>
</tr>
<tr>
<td>GG</td>
<td>70 (70)</td>
<td>79 (79)</td>
<td>36 (69.2)</td>
<td>16 (100)</td>
<td>26 (83.9)</td>
<td>1 (100)</td>
<td></td>
</tr>
<tr>
<td>Allele model</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allele C</td>
<td>35 (17.5)</td>
<td>24 (12)</td>
<td>19 (18.3)</td>
<td>-</td>
<td>5 (8.1)</td>
<td>-</td>
<td>0.6</td>
</tr>
<tr>
<td>Allele G</td>
<td>165 (82.5)</td>
<td>176 (88)</td>
<td>85 (81.7)</td>
<td>32 (100)</td>
<td>57 (91.9)</td>
<td>2 (100)</td>
<td></td>
</tr>
<tr>
<td>Recessive model</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC + GC</td>
<td>30 (30)</td>
<td>21 (21)</td>
<td>16 (30.8)</td>
<td>-</td>
<td>5 (16.1)</td>
<td>-</td>
<td>0.059</td>
</tr>
<tr>
<td>GG</td>
<td>70 (70)</td>
<td>79 (79)</td>
<td>36 (69.2)</td>
<td>16 (100)</td>
<td>26 (83.9)</td>
<td>1 (100)</td>
<td></td>
</tr>
<tr>
<td>Dominant model</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>5 (5)</td>
<td>3 (3)</td>
<td>3 (5.8)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.6</td>
</tr>
<tr>
<td>GC + GG</td>
<td>95 (95)</td>
<td>97 (97)</td>
<td>49 (49.2)</td>
<td>16 (100)</td>
<td>31 (100)</td>
<td>1 (100)</td>
<td></td>
</tr>
<tr>
<td>HMGB1 steady-state serum</td>
<td>0.3 (0.1–3)</td>
<td>0.6 (0.1–85)</td>
<td>0.9 (0.2–62)</td>
<td>1 (0.2–66)</td>
<td>0.3 (0.1–85)</td>
<td>1.1</td>
<td>&lt; 0.05*</td>
</tr>
</tbody>
</table>

*P value from contingency table
†P value from t-test


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median of 3.2 ng/ml and a range of 0.3 to 76.4 ng/ml compared to the steady-state level with a median of 0.2 ng/ml and a range of 0.2 to 7.4 ng/ml, p < 0.0001 (Figure 4).

### Discussion

Regarding the rs2249825 genotype, the current study reported control frequencies agreed with frequencies reported in the 1000 genome project [25] and with Qiu et al. [26], who investigated the rs2249825 frequency distribution in sepsis patients. On the other hand, the frequencies were in clear contrast to those reported by Wang et al. [27] in Chinese newly diagnosed histologically confirmed colorectal carcinoma and their genetic match control. They reported additive genotype (GG, GC, and CC) as 3.75%, 20.42%, and 75.83%, respectively, in the control group. The discrepancy between the current study and the 1000 genome project reported frequency on the one hand and on the other hand, the study of Wang et al. [27] might be attributed to their relatively small sample size. They also failed to report the ethnic subgroup within their Chinese subjects, which were later proven in the literature to be of a significant contribution to the genetic polymorphisms. The current study rs2249825 reported frequencies in SCD are the first work, to the authors’ knowledge.

HMGB1 steady-state serum level results were concomitant with the results reported by Xu et al. [23], who examined pediatric SCD (Hb SS and
Hb S(β0 thalassemia) human patients. They detected a statistically increased steady-state serum HMGB1 in SCD human patients in comparison to their respective controls (p = 0.047). They did not analyze HMGB1 steady-state serum levels across the disease severity classes of Shah et al. [17].

The serum level of HMGB1 samples from patients with VOC in our study was in concordance with results detected by Xu et al. [23]. They detected a difference between the human SCD patients in their VOC state and their steady-state condition in regard to HMGB1 serum level, with higher VOC sample results (p = 0.036). This finding was also similar to that reported by Kalinina et al. [28] where they studied the intima of atherosclerotic plaques and compared it to the surrounding healthy intima, using immunohistochemistry, in postmortem samples from thoracic and abdominal aortas, not later than 6 hours after death, from 20 males and females, at the Russian Cardiology Research Complex, Moscow.

Although the paired steady-state and VOC samples of the SCD patients exhibited a 98.5% power of the study, paired sepsis samples, paired acute osteomyelitis, as well as paired venous thromboembolism samples, were not available during the study period. The current study elucidated the change in total HMGB1 protein level, while a more comprehensive study characterizing different HMGB1 isoforms by Electrospray Ionization–Liquid Chromatography-Tandem Mass Spectrometry (ESI-LC-MS/MS) would be more informative in terms of the possibility of fabrication of isoform-specific drug (i.e., a drug targeting the disulfide HMGB1 but not the fully oxidized isoforms).

Based on higher serum HMGB1 levels in the SCD patient group compared to the control group, higher levels in the Hb SS patient group compared to the control group, in addition to higher serum HMGB1 levels found during VOC compared to steady-state levels, we conclude that serum HMGB1 could be a potential therapeutic target in the management of SCD and its VOC state.

References


PMid:4774120

PMid:17610420


PMid:29200952

PMid:25339362

PMid:23719301


PMid:30423384

PMid:27665685

PMid:15374849