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Interleukin-1β and interleukin-6 gene polymorphisms in Egyptian sickle cell disease patients

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Abstract

Background: Sickle cell disease (SCD) is a disorder characterized by a heterogeneous clinical outcome. Interleukin-1β (IL-1β) and interleukin-6 (IL-6) are important mediators of inflammatory response. Genetic modifiers that alter cytokine levels may contribute to the clinical variability of SCD. The present study investigated the associations of IL-1β + 3954 C>T and IL-6 (− 174G>C and − 597 G>A) gene polymorphisms with clinical and laboratory data in SCD patients. The study was conducted on 100 SCD patients (59 sickle cell anemia patients "SS" and 41 sickle beta thalassemia patients "Sβ"). Fifty age- and sex-matched healthy volunteers were included as a control group. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique was used for the detection of IL-1β and IL-6 gene polymorphisms.

Results: The homomutant genotypes of IL-1β (+ 3954 C>T), IL-6 (− 174G>C), and IL-6 (− 597 G>A) were infrequently presented among SCD patients and control group. No significant differences were detected between SS, Sβ patients, and control group as regards the genotypic frequencies and allele distributions of the studied polymorphisms. As regards the clinical complications, the mutant genotypes of IL-1β (+ 3954 C>T) had a significantly higher frequency among Sβ patients with splenomegaly. Hemoglobin is significantly lower in SS patients with mutant allele (AA and GA) for IL-6 (− 597 G>A) ($P = 0.005$), while Sβ patients with mutant genotype for IL-6 (− 597 G>A) had significantly higher total leucocytic count ($P = 0.031$).

Conclusion: IL-1β (+ 3954 C>T), IL-6 (− 174G>C), and IL-6 (− 597G>A) polymorphisms are not associated with disease phenotype. However, IL6 polymorphism (− 597 G>A) might predispose to underlying inflammatory process.

Keywords: IL-1β (+ 3954 C>T), IL-6 (− 174G>C), IL-6 (− 597G>A), Polymorphism, Sickle cell disease

Background

Sickle cell disease (SCD) is a common hematological disorder that affects millions of people worldwide. It is characterized by chronic hemolysis and associated with recurrent infections leading to multiple painful crises [1]. These painful crises resulted in organ damage and subsequently early death pathophysiology of SCD including the vaso-occlusive crisis is a complex process. Interactions between sickle cells, immune cells, and vascular endothelial cells result in injuring the endothelium and lead to the production of pro-inflammatory cytokines and procoagulant mediators contributing to the disease pathogenesis [2–4]. Moreover, it is believed that these interactions are also activated sub-clinically during steady-state disease condition [5, 6].

SCD patients exhibit higher levels of several pro-inflammatory cytokines, including interleukin-1 beta (IL-1β), IL-6, IL-3, tumor necrosis factor α (TNF-α), granulocyte monocyte-colony stimulating factor (GM-CSF), endothelin-1, and prostaglandin E2 [7]. These cytokines are regulated by genetic factors that modify the susceptibility and severity of the disease [8].

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Three single-nucleotide polymorphisms (SNPs) have been reported in IL-1β, all representing C-T base transitions, at positions − 511, − 31, and + 3954 base pairs from the transcriptional start site. These polymorphisms have been associated with an increased risk of developing a number of inflammatory diseases and cancer [9]. In SCD, − 511 and + 3954 genetic polymorphisms of IL-1β have been claimed to predispose to vaso-occlusion via the inflammation, cellular adhesion, and coagulation processes [10].

The IL-6 gene is regulated mainly at the transcriptional level. Within its promoter, − 597G>A, − 572G>C, and − 174G>C SNPs have been described [11]. IL-6 cytokine has been implicated in regulation of acute phase reactants. It regulates the release of TNFα and the production of many adhesion molecules [12, 13].

The present study aimed to investigate IL-1β (+ 3954 C>T), IL-6 (− 174G>C), and IL-6 (− 597 G>A) genetic polymorphisms distribution in SCD versus the control group and to study their association with clinical and hematological data of SCD patients.

Methods

Participants

This case control study involved 100 SCD patients. They were diagnosed and followed up at the Pediatric Hematology Clinic of Children's Cairo University Hospital during the period from July 2016 till March 2017. Fifty age- and sex-matched healthy subjects were included in this study as a control group.

All SCD patients were in a steady state, which is defined as the period of time where the patient was not experiencing an acute painful crisis for at least four consecutive weeks after the last crisis. Patients who had infection or inflammatory condition at time of sampling or those who had been transfused within 3 months prior to the study time were excluded. Diagnosis of SCD patients was established using hemoglobin electrophoresis and high performance liquid chromatography. The ethics committee of the Faculty of Medicine, Cairo University, approved this study. Written informed consents were obtained from the participants' guardians before being involved in this study. The work has been carried out in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki) for experiments in human patients.

Medical records were reviewed for relevant patients' medical history, disease course, transfusion history, splenectomy, organomegaly, and history of painful crisis. Each patient had full clinical examination and laboratory investigations including complete blood picture, markers for hemolysis (serum bilirubin and lactate dehydrogenase (LDH)), and serum ferritin.

Detection of IL-1β (+ 3954 C>T), IL-6 (− 597 G>A), and IL-6 (− 174G>C) polymorphisms

Two milliliters of whole blood was collected from each patient and control by sterile venipuncture using a sterile ethylene diamine tetra-acetic acid (EDTA) vacutainer tube. Samples were stored at − 20 °C until used for DNA extraction.

DNA extraction was done using Quick-gDNA™ MiniPrep kit (Zymo Research, USA) according to the manufacturer's instructions. DNA amplification was done in a 25-μl reaction mixture using extracted DNA, MyTaq™ Red Mix (2x) (Bioline, UK), primers (Biosearch Technologies, USA) (Table 1), and nuclease-free water. The PCR cycle conditions for IL-1β (3954 C>T) were initial denaturation at 95 °C for 2 min followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 57 °C for 1 min, extension at 72 °C for 1 min, and a final extension step at 72 °C for 5 min. PCR cycle conditions for IL-6 (174G>C) were initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 45 s, and a final extension step at 72 °C for 5 min. As for IL-6 (597 G>A), PCR cycle conditions were initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 59 °C for 45 s, extension at 72 °C for 1 min, and a final extension step at 72 °C for 10 min. PCR products were digested with the corresponding restriction enzymes (RE) purchased from New England Biolabs (USA) according to the manufacturer's protocols (Table 1). Amplified products and restriction fragments were run on 2% agarose gel, stained with ethidium bromide (Fig. 1).

Statistical methods

Results are expressed as median and range or number and percentage as appropriate. Comparison between categorical data was performed using chi-square test. Fisher exact test was used instead when the expected frequency is less than 5. Test of normality, Kolmogorov-Smirnov test, was used to measure the distribution of data; accordingly, comparison between variables in the two groups was performed using either unpaired *t* test or Mann-Whitney test whenever it was appropriate. Comparison between the 3 groups was done using Kruskal-Wallis test (non-parametric ANOVA); then, multiple comparisons were done based on Kruskal-Wallis test. Odds ratio with 95% confidence interval (CI) was used for risk estimation. Statistical Package for Social Sciences (SPSS) computer program (version 21) was used for data analysis. $P < 0.05$ was considered significant.

Table 1 Primer sequences

Genetic polymorphism *Primer sequence (5′ -3′)*	Restriction enzyme	Product (bp)
[a]**IL-1β (+3954 C>T)** F-TC AGG TGT CCT CGA AGA AAT CAA A R-GCT TTT TTG CTG TGA GTC CCG	Taq[a]I	182
[b]**IL-6 (-174G>C)** F-TGA CTT CAG CTT TAC TCT TTG R-CTG ATT GGA AAC CTT ATT AAG	NlaIII	198
[c]**IL-6 (-597 G>A)** F-GGA GTC ACA CAC TCC ACC TG R-AGC AGA ACC ACT CTT CCT TTA CTT	FokI	419

[a]Ismaili et al. [14]
[b]Ghavimi et al. [15]
[c]Ambreen et al. [16]

Results

Clinical and demographic data of the studied groups

Patients included 46 females and 54 males. Their age ranged between 2 and 17 years with a median age of 11 years; positive consanguinity was present in 32% of patients. The SCD cases comprised 2 groups: 59 sickle cell anemia patients (SS) and 41 sickle beta thalassemia (Sβ) patients.

Among studied SCD patients, 39% of patients had splenomegaly, 24% had history of pulmonary hypertension, 8% experienced avascular necrosis, 2% had history of leg ulcers, 17% had history of gall bladder disease, 16% of the patients had splenectomy, and 6% had nephropathy.

As regards laboratory data of the sickle patient group, hemoglobin levels ranged between 6 and 11.7 mg/dl with a median of 8.7 mg/dl, and total leucocytic count (TLC) ranged between 3.4 and 27.5 × 10^9/l with a median of 9.3 × 10^9/l. Total bilirubin ranged from 0.3 to 10.4 mg/dl with median level of 1.7 mg/dl, while indirect bilirubin ranged from 0.2 to 10.3 with a median value of 1.40. Median LDH level was 342 U/l and ranged between 161 and 741 IU/l. As regards serum ferritin level at time of the study, it ranged from 46 to 4381 with a median value of 384 μg/l.

Fifty age- and sex-matched healthy subjects were included in the study as a control group. The control group included 22 (44%) females and 28 (56%) males.

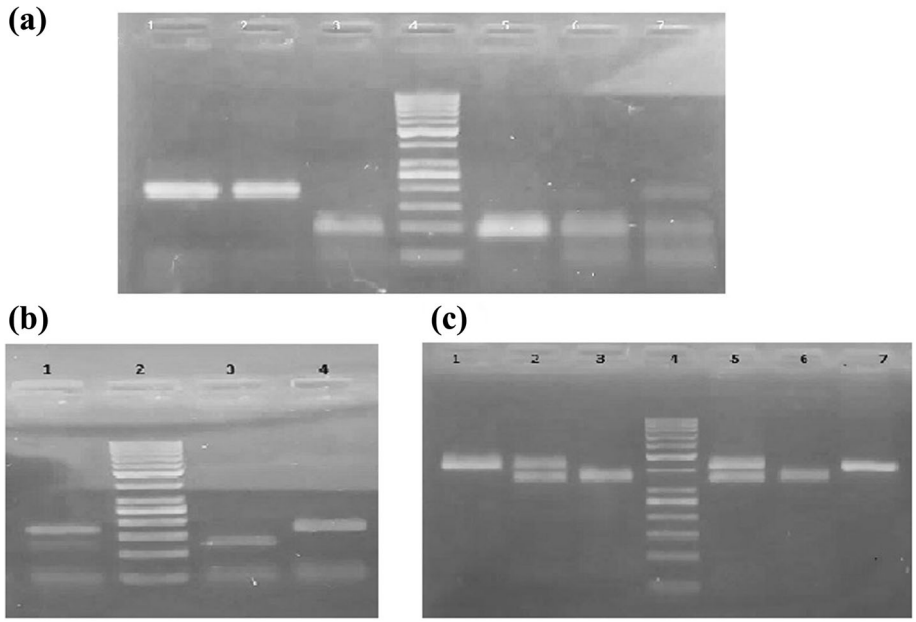

Fig. 1 PCR-RFLP amplification products. **a** Representative gel of IL-1β (+ 3954C>T) polymorphism. Lane 1 and 2: TT genotype (182 bp); lane 3, 5, and 6: CC genotype (97 and 85 bp); lane 7: CT genotype (182, 97, and 85 bp); lane 4: 50 bp DNA ladder (50, 100, 150, 200, 250, 300, 400, and 500). **b** Representative gel of IL-6 (– 174G>C) polymorphism. Lane 1: GC genotype (168, 119, and 49 bp); lane 3: CC genotype (119 and 49 bp); lane 4: GG genotype (168 and 30 bp); lane 2: 50 bp DNA ladder. **c** Representative of IL-6 (– 597G>A) genotypes: lane 1, 7: GG genotype (419 bp); lane 2 and 5: GA genotype (419, 362, and 57 bp); lane 3 and 6: AA genotype (362 and 57 bp); lane 4: 50 bp DNA ladder

Their age ranged between 2 and 15 years with a median age of 11 years.

IL-1β (+ 3954 C>T) polymorphism

Genotypic distribution and allele frequencies of IL-1β (+ 3954 C>T) polymorphism did not differ significantly between SCD patients and the control group ($P > 0.05$). TT mutant genotype was detected in 2 control subjects (4%) and in 5 SCD patients (5%), so subjects with either TT or CT genotype were considered as one group (Table 2). Similar frequencies were detected among the control, SS, and Sβ groups regarding mutant genotype (CT + TT) (56%, 57.6%, and 51.2% respectively, $P = 0.812$) and mutant T allele (30%, 32.2%, and 26.8% respectively, $P = 0.716$).

IL-6 (– 597 G>A) and (– 174 G>C) polymorphisms

No significant differences were detected between genotypic and allele frequencies of IL-6 (– 597 G>A) and IL-6 (– 174 G>C) among SCD patients when compared to the control group ($P > 0.05$) (Table 2). AA mutant genotype of IL-6 (– 597 G>A) was detected in one control subject (2%) and in 3 SCD patients (3%) while CC mutant genotype of IL-6 (– 174 G>C) was detected in one control subject out of 49 subjects (2%) and in 4 SCD patients out of 80 patients (4%). So subjects with either heterozygous or mutant genotypes were considered as one group for each polymorphism. On comparing the control, SS, and Sβ groups, similar frequencies were detected regarding the mutant genotype of IL-6 (– 174 G>C) polymorphism (24.5%, 27.7%, and 21.2% respectively, $P = 0.804$), mutant C allele (13.3%, 17%, and 10.6% respectively, $P = 0.498$), mutant genotype of IL-6 (– 597 G>A) polymorphism (20%, 25.4%, and 39% respectively, $P = 0.117$), and its mutant A allele (11%, 16.1%, and 19.5% respectively, $P = 0.272$).

Combined genotype

Combined genotype refers to the presence of at least two mutations out of the three studied interleukin polymorphisms in the same patient. On comparing SCD patients with the control group regarding combined genotypes, no significant difference was detected ($P = 0.523$).

Among the SS group, 30% harbored combined genotype, while in Sβ patients, 24% had combined genotypes and 22% of the control group showed combined genotypes ($P = 0.697$).

Relation between IL-1β (+ 3954 C>T), IL-6 (– 597 G>A), and IL-6 (– 174 G>C) and different clinical complications in sickle cell patients

The frequencies of avascular necrosis, pulmonary hypertension, cholileithiasis, and splenomegaly were higher among sickle patients (SS and Sβ) with mutant allele for IL-1β (+ 3954 C>T) than in patients with wild genotype, yet these differences did not reach a statistical significance (Table 3). However, Sβ patients with mutant allele (CT + TT) showed statistically significant higher frequency of splenomegaly (61.9%) than those with wild genotype (30%) ($P = 0.041$); other complications did not

Table 2 IL-1β (+ 3954 C>T), IL-6 (– 174 G>C), and IL-6 (– 597G>A) polymorphisms; genotype and allele frequencies in SCD patients and in the control group

Polymorphism	Control, ***N*** (%)	SCD patients, ***N*** (%)	Odds ratios (95% CI)	***P*** value[a]
IL-1β (+ 3954 C>T)				
Wild genotype (CC)	22 (44.0)	45 (45.0)	1.041 (0.526–2.062)	0.908
Mutant genotype (CT + TT)	28 (56.0)	55 (55.0)		
C Allele	70 (70.0)	140 (70.0)	1.000 (0.592–1.688)	1.000
T Allele	30 (30.0)	60 (30.0)		
IL-6 (– 174G>C)				
Wild (GG)	37 (75.5)	60 (75.0)	1.027 (0.450–2.344)	0.948
Mutant (GC + CC)	12 (24.5)	20 (25.0)		
G Allele	85 (86.7)	137 (85.6)	0.911 (0.438–1.894)	0.803
C Allele	13 (13.3)	23 (14.4)		
IL-6 (– 597G>A)				
Wild (GG)	40 (80.0)	69 (69.0)	0.556 (0.247–1.254)	0.157
Mutant (GA + AA)	10 (20.0)	31 (31.0)		
G allele	89 (89.0)	165 (82.5)	0.583 (0.282–1.203)	0.144
A allele	11 (11.0)	35 (17.5)		

[a]$P < 0.05$ is significant

Table 3 Relation between IL-1β (+ 3954 C>T), IL-6 (− 597 G>A), and IL-6 (− 174 G>C) and different clinical complications in sickle cell patients

	Avascular necrosis, ***N*** = 8	Pulmonary hypertension, ***N*** = 24	Cholelithiasis,***N*** = 17	Splenomegaly, ***N*** = 39
IL1ß (+ 3954C>T)				
Wild (CC)	3 (37%)	8 (33%)	7 (41%)	16 (41%)
Mutant (CT, TT)	5 (63%)	16 (66%)	10 (59%)	23 (59%)
P value	0.656	0.187	0.728	0.523
IL6 (− 174 G>C)				
Wild (GG)	5 /7(71%)	17/21 (81%)	13/16 (81%)	25/32 (78%)
Mutant (GC, CC)	2/7(29%)	4/21 (19%)	3/16 (19%)	7/32 (22%)
P value	0.819	0.463	0.518	0.501
IL-6 (− 597G>A)				
Wild (GG)	4 (50%)	20 (83%)	12 (71%)	28 (72%)
Mutant (GA, AA)	4 (50%)	4 (17%)	5 (29%)	11 (28%)
P value	0.226	0.081	0.876	0.628

differ significantly between different IL-1β genotypes in Sβ or in SS patients ($P > 0.05$).

The frequency of complications among IL-6 polymorphisms in the two sickle groups did not differ significantly in relation to different genotypes. Among our studied sickle patients, 2 patients had a history of leg ulcers and they harbored the mutant allele of the 3 studied polymorphisms.

Relation between IL-1β (+ 3954 C>T), IL-6 (− 597 G>A), and IL-6 (− 174 G>C) and different laboratory data in SS and Sβ patients

Hemoglobin was significantly lower in SS patients with mutant allele (GA, AA) for IL-6 (− 597 G>A) (median 7.7 mg/dl, range 6.0–9.4 mg/dl) than those with wild GG genotype (median 8.8 mg/dl, range 6.4–11.7 mg/dl) ($P = 0.005$), while Sβ patients with mutant genotype for IL-6 (− 597 G>A) had significantly higher TLC (median 10.5 × 10^9/l, range 4.8–17.7 × 10^9/l) than in Sβ patients with wild genotype (median 7.2 × 10^9/l, range 3.4–27.5 × 10^9/l) ($P = 0.031$). Other laboratory parameters and markers of hemolysis, total bilirubin, indirect bilirubin, and LDH did not differ significantly between different genotypes in SS patients and Sβ patients.

No statistical significance differences were detected between wild and mutant genotypes of IL1β (+ 3954C>T) and IL-6 (− 174 G>C) among SS and Sβ patients as regards different laboratory data, markers of hemolysis, and ferritin levels.

Discussion

Cytokines are involved in maintaining the balance between proinflammatory and anti-inflammatory stimuli in different conditions [17]. Microvascular occlusion in SCD, either clinical or subclinical, and hemolysis are crucial stimulators of cytokines and acute phase reactants production [7, 18]. On the other hand, cytokines seem to contribute in the pathogenesis of the vaso-occlusion crisis in SCD [18]. Among the cytokines that promote inflammation, IL1 β and IL6 are two important cytokines.

Frequencies of genotypic distribution of IL-1β (+ 3954C>T) among our control group were comparable to different studies: Vicari et al. [19], Ferreira et al. [20], and Chi et al. [17] who recorded TT as the least presented genotype. While in SS patients, IL-1β (+ 3954C>T) genotyping revealed that 42.4% had the wild CC genotype, 50.8% were heterozygous, and 6.8% were homozygous for TT. Vicari et al. [19] recorded frequencies of 54%, 38%, and 8% for CC, CT, and TT genotypes respectively among sickle cell disease patients. Both our study and Vicari et al. [19] study agreed that no statistically significant difference was detected between the genotypic frequencies among SS patients when compared to the control group. As regards Sβ patients, reported genotypic frequencies were similar to SS patients and the control group ($P > 0.05$). Up to our best knowledge, IL-1β gene polymorphism was not investigated before in Sβ per se. Recently, Khorshied et al. [21] detected no significant differences in IL-1β genotype frequencies among 84 Egyptian SCD children including 24 Sβ patients when compared to a control group.

Our results suggested that IL-1β (+ 3954C>T) polymorphism has no significant impact on the complications or the hemolytic process in sickle patients. Although the mutant genotype was more frequent among sickle patients who experienced complications, yet this difference did not reach a statistical significance. This is in contrast to Vicari et al. [19] who found that the presence of at least one T allele of IL-1β (+ 3954C>T) was associated with significantly higher frequency of osteonecrosis suggesting the role of T allele in

predisposition to vaso-occlusive process. Further studies on a larger cohort group are required for proper evaluation of the role of IL-1β in sickle cell disease patients.

IL-6 is a multifunctional cytokine with a biovital role in many inflammatory and immunological processes. Increased levels of IL-6 in SCD may represent contribution in exacerbation of severity of the disease [22]. The promoter – 174 polymorphism of the IL-6 gene is claimed to be related to IL-6 gene transcription rate and its circulating level. This has allowed this SNP to be used as a genetic instrument to probe the causality of higher IL-6 levels in many different diseases [23].

As regards IL-6 (– 174G>C) and IL6 (– 597G>A) genotypic distributions in the control and sickle patient groups, results revealed that the Egyptian population had a rare – 174 CC and – 597 AA mutant genotypes, while the wild homozygous variants were the most frequent genotypes in both groups. These frequencies are similar to what was detected in different studies with different ethnic groups [19, 21, 24–26].

There was no statistically significant difference in IL-6 (– 174G>C) and IL6 (– 597G>A) genotypic frequencies between SS and Sβ patients and control groups ($P > 0.05$). This is in accordance with Vicari et al. [19] who reported similar genotypic frequencies between SS patients and the control group. Up to our best knowledge, no previous results were published for IL-6 (– 174G>C) and IL6 (– 597G>A) polymorphisms in Sβ patients.

Different clinical complication frequencies among sickle patients did not differ significantly between those with wild and those with mutant IL-6 genotypes for both polymorphisms. Higher levels of IL-6 protein in SCD were documented in several studies such as Elzubeir et al. [22] who found a significant association between elevation in serum IL-6 and development of complications in SCD and Bourantas et al. [27] who found a statistically significant elevation of IL-6 concentrations among patients of steady SCD. However, little research links the IL-6 polymorphisms with SCD patients. Vicari et al. [19] reported inconsistent influence of IL-6 (– 174G>C) and IL6 (– 597G>A) polymorphisms on cholelithiasis, stroke, retinopathy, and leg ulcers according to the different assessed genetic models: recessive, dominant, and codominant models. Correlation between the different genetic variants and the level of IL6 is recommended to elucidate its contributions to pathogenesis of sickle cell disease complications.

On comparing laboratory data between patients with wild and those with mutant genotype for IL-6 (– 597 G>A) polymorphism, hemoglobin level was significantly lower in SS patients with mutant genotype ($P = 0.005$) and TLC level was significantly higher in the Sβ patients with mutant genotype ($P = 0.031$). SCD patients often had a high reticulocyte count as a consequence of chronic hemolysis. Reticulocytes bind to plasma and endothelial fibronectin, and endothelial vascular cell adhesion molecule (VCAM-1) via very late antigen-4 (VLA-4) [28, 29]. These interactions are augmented by inflammatory cytokines such as TNF-α and IL-1 [6]. Moreover, growing body of evidence indicates that leucocytes may initiate and propagate the vaso-occlusive crisis in sickle cell patients. In addition, the high leucocytic count during steady state may contribute to vascular complications. Also, it is postulated that vascular endothelium activation promotes leukocyte recruitment, activation, and adhesion, augmenting the interactions between erythrocyte and adherent leucocytes [6, 30].

Conclusion

This study did not detect a significant relationship between IL-1β (+ 3954C>T), IL-6 (– 174G>C), and IL-6 (– 597G>A) genetic polymorphisms and SCD phenotypes. However, IL-6 (– 597G>A) polymorphism might predispose to underlying inflammatory process and subsequent complications. Rarity of mutant alleles of the IL-1β and IL-6 polymorphisms among our studied population hampered reaching a definite conclusion on their influence on the disease status. Investigation of larger cohort of patients is recommended to elucidate their proper role in sickle cell disease.

Abbreviations

SCD: Sickle cell disease; IL: Interleukin; PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism; TNF-α: Tumor necrosis factor α; GM-CSF: Granulocyte monocyte-colony stimulating factor; SNP: Single-nucleotide polymorphisms; LDH: Lactate dehydrogenase; TLC: Total leucocytic count; VCAM-1: Vascular cell adhesion molecule; VLA-4: Very late antigen-4

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Authors' contributions

YE carried out the molecular genetic studies, collected and analyzed the data, and drafted the manuscript. NS and HA carried out the genetic analysis, analyzed the data, and wrote the manuscript. ME designed the study, managed SCD patients, analyzed the data, and helped to draft the manuscript. SZ designed the study, analyzed the data, and revised the paper critically for its intellectual content. The authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon request.

Ethics approval and consent to participate

The ethics committee of the Faculty of Medicine, Cairo University, approved this study. Written informed consents were obtained from the participants' guardians before being involved in this study. The work has been carried out in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki) for experiments in human patients

Consent for publication

Not applicable.

Competing interests
The authors declare that they have no competing interests.

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