CLINICAL RESEARCH ARTICLE

Polymorphisms of the receptor for advanced glycation end products as vasculopathy predictor in sickle cell disease

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BACKGROUND: The genetic variants of the receptor for advanced glycation end products (RAGE) gene have been associated with vascular disease risk. The objective of this work was to explore the association of three single-nucleotide polymorphisms (SNPs) of RAGE gene (374T/A, 429T/C, and G82S) with vascular complications in SCD.
METHODS: The study was conducted on 40 children with SCD and 40 healthy children served as controls. All participants were genotyped for the three studied RAGE polymorphisms by polymerase chain reaction (PCR).
RESULTS: Regarding 374T/A polymorphism, the frequency of TA, TT genotypes and T allele were higher in patients ($p < 0.001$). T allele was associated with higher incidence of sickling crisis and stroke ($p < 0.05$). In the subgroup analyses of 429T/C polymorphism, an association between C allele and SCD vascular complications was observed ($p < 0.05$). Concerning the frequency of G82S genotypes of RAGE, GG variant was detected in 39 (97.5%) of the patients, as compared with 40 (100%) of controls ($p = 0.3$). A regression analysis proved that HbS%, serum ferritin, and the −374T and 429C alleles were significant independent predictors of frequent sickling episodes ($p < 0.05$).
CONCLUSIONS: The C allele of −429T/C and T allele of 374T/A RAGE polymorphisms may be considered as predictors for vascular dysfunction in SCD.

IMPACT:

- The C allele of −429T/C and T allele of 374T/A RAGE polymorphisms may be considered as predictors for vascular dysfunction in SCD patients.
- To our knowledge, our study is the first exploring the association of three single-nucleotide polymorphisms of RAGE gene (374T/A, 429T/C, and G82S) with vascular complications in SCD.
- Early identification of patients carrying these genetic variants might be of great importance not only to identify subjects at risk of vasculopathy but also to direct them to RAGE-targeted treatments.

INTRODUCTION

Sickle cell disease (SCD) is characterized by lifelong continuous oxidative stress. A high oxidative burden in SCD is caused by factors, such as increased intravascular hemolysis, ischemia–reperfusion injury, and chronic inflammation.[1]

The oxidative stress and chronic inflammation are potentially contributing to SCD-related vasculopathy, which has been implicated in the development of secondary disease states, such as stroke, acute chest syndrome and other chronic organ damage leading to decreased lifespan and poor quality of life.[2]

Oxidative stress in SCD potentially enhances the generation and accumulation of advanced glycation end products (AGEs).[3] They are a heterogeneous group of biochemical modifications that result from non-enzymatic glycation and oxidation of proteins and lipids.[4] AGEs, which are well-established markers of oxidative stress, have several pathological mechanisms that could contribute to organ damage and disease severity.[5]

AGE's deleterious effects are mediated by their cellular receptor RAGE (receptor for advanced glycation end products), which is a ubiquitous receptor present on epithelial, inflammatory, and vascular cells. It is usually expressed in high levels at the sites of vascular injury.[6]

Upon AGE–RAGE binding, RAGE activates multiple downstream cellular signaling cascade, gene expression, and production of pro-inflammatory mediators and free radicals resulting in enhanced oxidative stress and persistent RAGE hyperexpression, thereby converting a brief pulse of cellular activation to sustained cellular perturbation, local inflammation, and tissue damage.[7]

There is increasing evidence that links RAGE axis hyperactivities to the pathobiology of tissue damage in many vascular diseases.[8] This has evoked a focus of attention to the potential of RAGE gene as an attractive candidate gene for vascular complications and the genetic variants affecting RAGE expression as an important disease marker for identifying subjects at risk for vascular disease.[9]

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The gene for RAGE is located on chromosome 6p21.3 and comprises 11 exons. To date, numerous genetic variants have been identified in the RAGE gene, the majority of which are single-nucleotide polymorphisms (SNPs) affecting its expression and signaling.[10]

A significant body of literature points to the importance of three SNPs that have been identified in the RAGE gene: 374T/A, 429T/C, and G82S polymorphisms. In the promoter region, it has been reported that the substitution of T to A nucleotide in 374T/A polymorphism results in abolition of a nuclear protein-binding site that is probably repressing RAGE transcription. In addition, the substitution of T to C nucleotide in 429T/C variant affects transcription factor binding and increasing transcription of RAGE.[11]

The G82S polymorphism is the only frequent coding change in the ligand-binding domain in exon 3. This variant exhibits an enhanced cell surface expression, ligand affinity, and pro-inflammatory signaling.[12]

Recently, RAGE polymorphic variants have attracted considerable interest and were associated with vasculopathies in different pathological states.[6] To date, no study has been designed to test the hypothesis that RAGE gene polymorphisms may be involved in the vascular pathology of SCD. Accordingly, we investigated three common SNPs of RAGE gene: 374T/A, 429T/C, and G82S in SCD, and analyzed the association of these genetic markers with vascular complications in children and adolescents with SCD.

PATIENTS AND METHODS
This case–control cross-sectional study included 40 Egyptian children and adolescence with SCD recruited from the regular attendants of the Pediatric Hematology Clinic, Pediatric Hospital, Ain Shams University. Forty age- and sex-matched healthy subjects were enrolled as a control group.

Hemoglobin analysis was done using high-performance liquid chromatography (HPLC) and confirmed by genotyping.[13,14] Out of the 40 patients, 26 patients (65.0%) were diagnosed as sickle cell anemia and 14 patients (35.0%) were diagnosed as sickle cell beta thalassemia. An informed consent was obtained from the guardian of each patient or control before participation. The procedures were approved by the Ethical Committee of Human Experimentation of Ain Shams University and are in accordance with the Helsinki Declaration of 1975.

Exclusion criteria were infection; chronic inflammatory condition other than SCD; complications unrelated to SCD, such as cardiovascular diseases, rheumatoid arthritis or other autoimmune diseases; diabetes mellitus (DM); or renal insufficiency.

At the time of sample collection, all patients were in a steady state (defined as a period without pain or painful crisis for at least 4 weeks)[15] and those who had sickling crisis (defined as musculoskeletal pain that could not be explained except by SCD)[16] were excluded. The frequency of sickling crisis in the previous year was divided into mild (defined as ≤2 episodes requiring medical visits) or severe (defined as ≥3 episodes requiring medical visits).[17] Acute chest syndrome was defined as a new radiodensity on chest radiograph accompanied by fever and/or respiratory symptoms.[18] Diagnosis was confirmed by the presence of radiographic changes in chest X-ray. Stroke was manifested by either seizures or signs of lateralization or disturbed conscious level. Diagnosis based on magnetic resonance imaging of the brain and whether stroke was arterial or venous was determined by magnetic resonance angiography and magnetic resonance venography of the brain.

All of SCD patients were transfused. Chronic transfusion protocols were implemented in cases with previous history of stroke or acute chest syndrome as a prophylactic measure. The transfusion was on monthly basis, while the transfusion in other patients was on demand, based on individual patient basis.

All of the patients received hydroxyurea (Bristol-Meyers-Squibb, NY, USA) as an oral daily dose ranging from 10 to 25 mg/kg/day.

The patients with serum ferritin >1000 µg/L received the three main chelator agents as follows: deferoxamine (Novartis, Basel, Switzerland) as a subcutaneous injection over 8 h ranging from 20 to 50 mg/kg, deferasirox (Novartis, Basel, Switzerland) as a single oral dose ranging from 20 to 40 mg/kg, and deferiprone (Apotex, Toronto, Canada) orally divided into 2 or 3 doses ranging from 50 to 100 mg/kg.

Sample preparation
Peripheral blood samples were collected on potassium–ethylene diamine tetra-acetic acid (1.2 mg/mL) for complete blood count (CBC) and hemoglobin analysis. For PCR, samples were kept at −20 °C until used for DNA extraction. For chemical analysis, clotted samples were obtained and serum was separated by centrifugation for 15 min at $1000 \times g$.

Laboratory analysis
Laboratory investigations included CBC using Sysmex XT-1800i (Sysmex, Kobe, Japan), examination of Leishman-stained smears for differential white blood cell count, hemoglobin analysis by HPLC using D-10 (BioRad, Marnes La Coquette, France), markers of hemolysis (lactate dehydrogenase and indirect bilirubin), and serum ferritin on Cobas Integra 800 (Roche Diagnostics, Mannheim, Germany).

Genomic DNA extraction
Total DNA was extracted from whole blood using the QIAamp® kits in accordance with the manufacturer's instructions (Qiagen, Germany). Released DNA was bound exclusively to the biospin membrane in the presence of binding buffer. After several washing procedures, the DNA is then eluted from the membrane prior to the genotyping assays. For long-term storage, the eluted DNA in buffer was kept at −20 °C until amplification.

RAGE variant genotyping
Genotyping for each subject was performed using the TaqMan SNP assays (Applied Biosystems, CA, USA). Genotyping of RAGE SNPs: 374T/A (rs1800624), 429T/C (rs1800625), and G82S (rs2070600), was carried out on a real-time PCR instrument (Qiagen, Rotor-Gene Q, Germany) according to the manufacturer's protocol (95 °C for 10 min and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min). After PCR amplification, a post-PCR plate reads were carried out to generate allelic discrimination plot.

Statistical analysis
Data were collected, revised, coded, and entered to the Statistical Package for Social Science (IBM SPSS™) version 20. Qualitative data were presented as numbers and percentages, while quantitative data were entered into Kolmogorov–Smirnov test of normality and parametric distribution data were presented as mean, standard deviations, and ranges, while non-parametric distribution data were presented as median with interquartile range. In order to compare the parametric quantitative variables between two groups, Student t test was applied. For comparison of non-parametric quantitative variables between the two groups, Mann–Whitney test was used. Comparison between the two groups with qualitative data was done using Chi-square test. A logistic regression analysis was employed to determine the predictors of frequent sickling crisis in the studied patients. A p value <0.05 was considered significant in all analyses. Chi-squared test was used to evaluate the Hardy–Weinberg equilibrium for the distributions of the allelic and genotypic frequencies of the studied SNPs.

RESULTS
The demographic, clinical and laboratory characteristics of the studied patients are shown in Table 1.

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Table 1. Descriptive data of the studied patients.

Parameters	Patients, $N = 40$	Control, $N = 40$
Age (years)		
Mean ± SD	9.25 ± 4.52	10.08 ± 3.35
Sex, n (%)		
Males	17 (42.5%)	19 (47.5%)
Females	23 (57.5%)	21 (52.5%)
Sickle crisis (attacks/year)		
<3	17 (42.5%)	—
≥3	23 (57.5%)	
History of stroke, n (%)		
Negative	31 (77.5%)	40 (100%)
Positive	9 (22.5%)	0 (0%)
History of acute chest syndrome, n (%)		
Negative	35 (87.5%)	40 (100%)
Positive	5 (12.5%)	0 (0%)
Hemoglobin, (g/dL)		
Mean ± SD	8.48 ± 1.54	12.43 ± 0.94
HbS% at study		
Median (IQR)	59.1 (38.65–70.55)	0
HbF% at study		
Median (IQR)	8.10 (1–13.65)	0.83 ± 0.14
LDH (mg/dL)		
Mean ± SD	459.83 ± 172.08	—
Indirect bilirubin (mg/dL)		
Mean ± SD	1.36 ± 0.72	—
Ferritin (µg/L)		
Median (IQR)	395.00 (268.5–1529.5)	—

Table 2. Genotypic distribution and allele frequency of the three assessed polymorphisms in the RAGE gene among the patients and control groups.

Genotypes	Control group $N = 40$	Patient group $N = 40$	Test value[a]	p Value
374T/A				
TT	2 (5.0%)	3 (7.5%)	7.376	**0.025**
TA	10 (25.0%)	21 (52.5%)		
AA	28 (70.0%)	16 (40.0%)		
Alleles				
T	14 (17.5%)	27 (33.75%)	5.542	**0.018**
A	66 (82.5%)	53 (66.25%)		
429T/C				
TT	32 (80.0%)	29 (72.5%)	0.748	0.688
TC	6 (15.0%)	9 (22.5%)		
CC	2 (5.0%)	2 (5.0%)		
Alleles				
T	70 (87.5%)	67 (83.7%)	0.457	0.499
C	10 (12.5%)	13 (16.3%)		
G82S				
GG	40 (100.0%)	39 (97.5%)	1.013	0.314
GS	0 (0.0%)	1 (2.5%)		
SS	0 (0.0%)	0 (0.0%)		
Alleles				
G	80 (100.0%)	79 (98.7%)	1.006	0.315
S	0 (0.0%)	1 (1.3%)		

[a]Chi-square test.
Bold values are significant ($p < 0.05$).

Analysis of RAGE gene polymorphisms in the study population
The genotype distributions of the three studied SNPs were examined in cases and controls. No deviation ($p > 0.05$) from Hardy–Weinberg equilibrium in both the case and control groups was achieved for the three studied polymorphisms. The genotype distributions and allele frequencies of the examined SNPs in RAGE gene between cohorts are presented in Table 2.

There was a significant difference in RAGE −374T/A genotype distribution between the SCD patients and the controls ($p = 0.025$), where the TA and TT genotype frequencies were higher in the patients compared to controls being 52.5%, 7.5% versus 25%, 5%, respectively. Moreover, T allele displays a higher frequency among the sickle patients ($p = 0.018$).

No differences were observed between the patients and the controls concerning the frequencies of the −479T/C and G82S genotypes of the RAGE ($p > 0.05$). The GG homozygote variant of G82S polymorphism was detected in 39 (97.5%) of the patients, as compared with 40 (19%) of healthy controls. In addition, the G allele was the major expressed allele among cases and controls, with no statistical difference between the two groups ($p > 0.05$).

Association between RAGE −347T/A genotypes and clinic laboratory characteristics of SCD patients
To explore the potential association between RAGE −347T/A genotypes and clinic laboratory characteristics of SCD patients, a subanalysis of the patients according to their genotypes was done. This analysis revealed that patients with TA and TT genotypes had higher levels of HbS% ($p = 0.038$) and were more prone to develop sickling crisis and stroke ($p = 0.006$ and 0.005, respectively) compared to their counterparts carrying the genotype AA (Table 3).

Association between RAGE −429T/A genotypes and clinic laboratory characteristics of SCD patients
Regarding the different genotypes of RAGE −429T/A, Table 3 revealed that genotypes with the variant C allele (CC and TC) were significantly associated with positive history of SCD-related vasculopathy, including sickling crisis, stroke, and acute chest syndrome ($p = 0.008$, 0.03, and 0.005, respectively). Notably, serum ferritin level was significantly higher among SCD patients carrying the variant C allele compared to non-carriers ($p = 0.01$).

Besides genotype analysis, we performed logistic regression model to obtain information about predictors of frequent sickling crisis in the studied patients. Our results proved that C allele of −429T/C polymorphism and T allele of 374T/A polymorphism together with HbS% and ferritin levels could be considered as significant independent predictors of frequent sickling crisis (Table 4).

DISCUSSION
The RAGE and its ligand AGEs are intimately involved in the pathobiology of a wide range of diseases that share common features, such as enhanced oxidative stress, inflammatory responses, and altered cell function.[19]

Enhanced AGE generation coupled with RAGE hyperactivity was revealed as a critical pathway involved in many vascular complications.[20] During the past two decades, numerous studies have proved the enhanced generation of AGEs in SCD[3] suggesting that they might be implicated in vascular damage and chronic organ complications.[5,21] Despite this progress in AGE studies, the functional importance of RAGE in SCD vasculopathy still remains to be fully realized.

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Table 3. Comparison of clinical and laboratory characteristics between RAGE −374A/T and RAGE −429T/C genotypes.

Parameters	−374T/A polymorphism			−429T/C polymorphism		
	TT, TA	AA	*p* Value	TT	TC, CC	*p* Value
Sickle crisis (attacks/year)						
<3	6 (25.0%)	11 (68.8%)	**0.006**[a]	16 (55.2%)	1 (9.1%)	**0.008**[a]
≥3	18 (75.0%)	5 (31.3%)		13 (44.8%)	10 (90.9%)	
History of stroke, *n* (%)						
Negative	15 (62.5%)	16 (100.0%)	**0.005**[a]	25 (86.2%)	6 (54.5%)	**0.032**[a]
Positive	9 (37.5%)	0 (0.0%)		4 (13.8%)	5 (45.5%)	
History of acute chest syndrome, *n* (%)						
Negative	20 (83.3%)	15 (93.8%)	0.329[a]	28 (96.6%)	7 (63.6%)	**0.005**[a]
Positive	4 (16.7%)	1 (6.3%)		1 (3.4%)	4 (36.4%)	
Hemoglobin (g/dL)						
Mean ± SD	8.57 ± 1.56	8.33 ± 1.53	0.635[b]	8.76 ± 1.68	7.72 ± 0.67	0.054[b]
HbS% at study						
Median (IQR)	63.6 (51–75.22)	41.5 (28.85–68)	**0.038**[c]	61.9 (44.7–71.1)	43 (29.2–64)	0.102[c]
HbF%						
Median (IQR)	6.3 (0–12.1)	9.06 (3.05–17.6)	0.207[c]	9.01 (0–15)	6.20 (2.5–12.2)	0.951[c]
LDH (mg/dL)						
Mean ± SD	468.79 ± 183.21	446.38 ± 158.75	0.692[b]	447.07 ± 173.22	493.45 ± 172.49	0.454[b]
Indirect bilirubin (mg/dL)						
Mean ± SD	1.49 ± 0.72	1.16 ± 0.69	0.163[b]	1.33 ± 0.72	1.44 ± 0.73	0.674
Ferritin (µg/L)						
Median (IQR)	571.95 (302–1724.5)	357.4 (229.15–1419)	0.204[c]	339 (250–1125)	1438.00 (742–3318)	**0.018**

[a]Chi-square test.
[b]Independent *t* test.
[c]Mann–Whitney test.
Bold values are significant (*p* < 0.05).

Table 4. Logistic regression analysis for predictors of frequent sickling crisis.

Parameters	Odds ratio (OR)	95% CI for OR		*p* Value
		Lower	Upper	
HbS% at study	1.041	1.005	1.079	0.025
429T/C gene polymorphism	12.308	1.388	109.097	0.024
374T/A gene polymorphism	6.600	1.621	26.871	0.008
Ferritin (µg/L)	1.003	1.001	1.005	0.012

In the light of these findings, we proposed the hypothesis that SCD may be considered as one of the conditions characterized by ligand–RAGE axis hyperactivity, which drives the strength and maintenance of cellular perturbation, tissue injury, and vasculopathy.

At a genetic standpoint, RAGE polymorphisms may represent new insights into the putative connection of genetically determined hyperactivities of the RAGE axis and susceptibility to RAGE-mediated pathogenesis.[6]

Although there have been associative studies of RAGE polymorphism with vascular complications in various pathological conditions, the associations of these polymorphisms with SCD vasculopathy remains unveiled.

This work studied two functional polymorphisms in the promoter region, −374T/A and −429T/C, as well as one of the coding change polymorphisms of RAGE, the G82S variant. The −374T/A polymorphism has been shown to be the most associated with vascular disease.[9] This SNP is involved in RAGE gene regulation by influencing the binding affinity of the transcription factor site.[22] Our results revealed a significant difference in genotype distribution and allele frequency in 374T/A polymorphism between SCD patients and control group. Interestingly, a novel finding of the present study was that SCD patients with AA genotype had lower levels of HbS% and so lower rates of hemolysis and autoxidation of HbS. Therefore, those patients were less prone to develop sickling crisis and stroke compared to TT, TA genotypes.

This result is consistent with the studies of Pettersson-Fernholm et al.,[23] Picheth et al.,[24] Lu and Feng,[25] and Chawla et al.[26] who found a protective role of −374A allele against macrovascular and microvascular complications of DM.

In attempts to explain the protective effect of the A allele, previous studies published by Falcone and his colleagues reported that AA genotype (374T/A) caused by the conversion of T allele to A allele leads to repression of RAGE expression, resulting in inefficiency in cellular signaling.[27–29]

Conversely, in other studies A allele was defined as a risk factor rather than a protective factor against diabetic complications. Their observation was attributed to the reduced binding of a nuclear factor to a regulatory element of the RAGE gene promoter enhancing RAGE transcriptional activity.[11,30]

Other researchers reported a non-significant association between −374T/A polymorphism and diabetic complications in various ethnic populations.[31,32]

Proximal to the −374T/A polymorphism is the −429T/C variant that is located upstream to 374T/A in the RAGE gene promoter. It is hypothesized that this SNP is involved in increased RAGE expression.[22]

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Ligand engagement of the hyperexpressed RAGE triggers its activation. This enhanced activation may increase reactive oxygen species generation and upregulate adhesion molecules and pro-inflammatory and prothrombotic mediators.[33] These concepts may lie behind the significant association between the 429C allele and the SCD-related vascular events among our SCD group.

It is well known that iron may contribute directly to endothelial damage and vasculopathy,[34] and previous reports done by Ballas et al.[35] and Vichinsky et al.[36] suggest an association between iron overload and organ dysfunction in chronically transfused patients. Therefore, the significantly elevated ferritin levels among our patients with the C allele may add to their risk of SCD-related vasculopathy.

The association of RAGE −429T/C gene variant with vascular complications has been also dealt with by many researches before; some confirmed the association, while others denied it.

A number of publications have revealed a role for the 429T/C variant as a marker for the diabetic and pre-diabetic states.[37,38] Similarly, a preliminary meta-analysis has reported that CC genotype (429T/C) might contribute to the susceptibility of diabetic nephropathy and coronary artery disease in patients with type 2 DM.[7,39] Consistent with these findings, Hudson et al.[11] noticed a significant increase of the C allele in diabetic retinopathy.

In contrast, three preliminary studies conducted on patients of different ethnic origins showed no significant association between −429T/C and vascular complications in type 2 DM.[31,40,41]

Indeed, many factors may contribute to the discrepancies between studies. First, ethnic origins of the population. Second, study design or the size of the study groups and/or the difference in other risk factors for vascular diseases. Third, the use of single locus association approach cannot explain multifactorial vascular disease.[42,43]

A significant body of literature points to the importance of the G82S polymorphism because it occurs in the ligand-binding V-domain of RAGE, which strongly suggests higher ligand affinity and increased generation of inflammatory mediators.[44]

Among all of our studied subjects, G allele of G82S polymorphism was the major expressed allele compared to S allele, with no statistically significant difference among cases and control group. The S allele frequency among our studied Egyptian subjects was similar to that detected in other studies conducted on European and African populations.[27] Conversely, higher frequencies of S allele were observed in Asian population,[45] especially in Chinese population.[46]

The differences in the findings published in individual studies are hindered by the fact that individual populations in the world can differ in the frequencies of the alleles of appropriate polymorphisms.

The most problematic feature of SCD is sickling crisis, which is an important index of disease severity. Although the vulnerability to sickling crisis among patients with SCD may be genetically predetermined on the basis of genotype variability, nonetheless, it is usually associated with a wide spectrum of risk factors.[47]

Our understanding of these factors and their pathophysiologic mechanisms is important to identify patients at risk of sickling crisis. Therefore, a regression analysis model was designed to determine the independent predictors of frequent sickling crisis.

It has been known that patients with high levels of HbS have higher rates of hemolysis due to higher autoxidation of HbS. During hemolytic episodes, the released free heme depletes NO leading to vasomotor dysfunction, slowing of blood, and activation of endothelium to a pro-adhesive state that promotes sickle vaso-occlusion. These observations are further supported by ours reporting HbS% as independent predictors of recurrent painful episodes.[48]

Moreover, a previous report suggested an association between iron overload and vasculopathy as well as an inflammatory response.[34] Inflammatory cytokines promote adhesion of deformable red cells to vascular endothelium contributing to the development of sickling crisis.[1] In the light of this concept, our finding, which reported ferritin level as a risk factor influencing frequent sickling crisis, could be explained. However, our finding needs to be strengthened by considering relevant confounders that affect ferritin level such as the frequency of blood transfusion and adherence to iron chelators.

When the promoter region variants of RAGE gene were inserted in a regression analysis model, the −374T and −429C alleles were strongly related to higher rates of sickling crisis. These two alleles have been associated with increased RAGE gene expression, which in turn alters the redox balance, upregulates various adhesion molecules, and amplifies pathological processes of vaso-occlusion.[33]

Taken together, our study reported HbS%, ferritin level, and 374T and 429C alleles of RAGE gene polymorphism as predictors of frequent sickling episodes. The inclusion of these predictors in clinical practice might improve early identification and management of at-risk SCD patients.

In conclusion, this study suggested that C allele of −429T/C and T allele of 374T/A RAGE polymorphisms may be considered as predictors for vascular dysfunction in SCD patients. Early identification of patients carrying these variants might be of great importance not only to identify subjects at risk of vasculopathy but also direct them to RAGE-targeted treatments such as antibodies that block the activation of RAGE signaling.

The main limitation of the current study is the small sample size. Larger population-based studies are necessary to verify the role of RAGE gene polymorphism in SCD. Moreover, future investigations are definitely required to investigate the role of RAGE polymorphisms in other SCD vasculopathies (i.e., sickle nephropathy). This will be the next important step in defining RAGE polymorphisms as genetic vasculopathy markers and turning their discovery into novel therapeutics.

AUTHOR CONTRIBUTIONS

ADDITIONAL INFORMATION

REFERENCES

1. Nur, E. et al. Oxidative stress in sickle cell disease; pathophysiology and potential implications for disease management. *Am. J. Hematol.* **86**, 484–489 (2011).
2. Chirico, E. N. & Pialoux, V. Role of oxidative stress in the pathogenesis of sickle cell disease. *IUBMB Life* **64**, 72–80 (2012).
3. Kashyap, L. et al. Sickle cell disease is associated with elevated levels of skin advanced glycation endproducts. *J. Pediatr. Hematol. Oncol.* **40**, 285–289 (2018).
4. Ahmed, N. Advanced glycation end products role in pathology of diabetic complications. *Diabetes Res. Clin. Pract.* **67**, 3–21 (2005).
5. Nur, E. et al. Plasma level of advanced glycation end products are associated with hemolysis-related organ complications in sickle cell patients. *Br. J. Hematol.* **151**, 62–69 (2010).

6. Mungun, D. et al. The relation between rage polymorphism and cardiovascular diseases: a review. *MOJ Gerontol. Ger.* **2**, 225–230 (2017).

7. Shi, Z., Lu, W. & Xie, G. Association between the RAGE gene-374T/A,-429T/C polymorphisms and diabetic nephropathy: a meta-analysis. *Ren. Fail.* **37**, 751–756 (2015).

8. Kouidrat, Y. et al. Advanced glycation end products and schizophrenia: a systematic review. *J. Psychiatr. Res.* **66**, 112–117 (2015).

9. Kalea, A. Z., Schmidt, A. M. & Hudson, B. I. RAGE: a novel biological and genetic marker for vascular disease. *Clin. Sci.* **116**, 621–637 (2009).

10. Tripathi, A. K. et al. Association of RAGE gene polymorphism with vascular complications in Indian type 2 diabetes mellitus patients. *Diabetes Res. Clin. Pract.* **103**, 474–481 (2014).

11. Hudson, B. I., Stickland, M. H., Futers, T. S. & Grant, P. J. Effects of novel polymorphisms in the RAGE gene on transcriptional regulation and their association with diabetic retinopathy. *Diabetes* **50**, 1505–1511 (2001).

12. Jang, Y., Kim, J. Y. & Kang, S. M. Association of the Gly82Ser polymorphism in the receptor for advanced glycation end products (RAGE) gene with circulating levels of soluble RAGE and inflammatory markers in nondiabetic and nonobese Koreans. *Metabolism* **56**, 199–205 (2007).

13. Wethers, D. L. Sickle cell disease in childhood: Part I. Laboratory diagnosis, pathophysiology and health maintenance. *Am. Fam. Phys.* **62**, 1013–1020 (2000).

14. Kwiatkowski, J. L. in *Manual of Pediatric Hematology and Oncology* 5th edn (ed. Lanzkowsky, P.) 221 (Elsevier, 2011).

15. van Beers, E. J. et al. Circulating erythrocyte-derived microparticles are associated with coagulation activation in sickle cell disease. *Haematologica* **94**, 1513–1519 (2009).

16. Platt, O. S. et al. Pain in sickle cell disease. Rates and risk factors. *N. Engl. J. Med.* **325**, 11–16 (1991).

17. Darbari, D. S. et al. Markers of severe vaso-occlusive painful episode frequency in children and adolescents with sickle cell anemia. *J. Pediatr.* **160**, 286–290 (2012).

18. Ballas, S. K. et al. Definitions of the phenotypic manifestations of sickle cell disease. *Am. J. Hematol.* **85**, 6 (2010).

19. Vazzana, N., Santilli, F., Cuccurullo, C. & Davi, G. Soluble forms of RAGE in internal medicine. *Intern. Emerg. Med.* **4**, 389–401 (2009).

20. Goldin, A., Beckman, J. A., Schmidt, A. M. & Creager, M. A. Advanced glycation end products; sparking the development of diabetic vascular injury. *Circulation* **114**, 597–605 (2006).

21. Somjee, S. S., Warrier, R. P., Thomson, J. L., Ory-Ascani, J. & Hempe, J. M. Advanced glycation end products in sickle cell anemia. *Br. J. Hematol.* **128**, 112–118 (2005).

22. Adams, J. N. et al. Genetic analysis of advanced glycation end products in the DHS MIND study. *Gene* **584**, 173–179 (2016).

23. Pettersson-Fernholm, K. et al. The functional -374 T/A RAGE gene polymorphism is associated with proteinuria and cardiovascular disease in type 1 diabetic patients. *Diabetes* **52**, 891–894 (2003).

24. Picheth, G., Costantini, C. O., Pedrosa, F. O., da Rocha Martinez, T. L. & de Souza, E. M. The −374A allele of the receptor for advanced glycation end products (RAGE) gene promoter is a protective factor against cardiovascular lesions in type 2 diabetes mellitus patients. *Clin. Chem. Lab. Med.* **45**, 1268–1272 (2007).

25. Lu, W. & Feng, B. The-374A allele of the RAGE gene as a potential protective factor for vascular complications in type 2 diabetes: a meta-analysis. *Tohoku J. Exp. Med.* **220**, 291–297 (2010).

26. Chawla, D. et al. RAGE gene polymorphism and expression: risk factor for vascular complications in type 2 diabetes mellitus. *Mol. Cytogenet.* **7**(S1), P29 (2014).

27. Falcone, C. et al. Relationship between RAGE gene polymorphism and angiographic coronary artery disease. *Int. J. Mol. Med.* **14**, 1061–1064 (2004).

28. Falcone, C. et al. -374T/A polymorphism of the RAGE gene promotor in relation to severity of coronary atherosclerosis. *Clin. Chim. Acta* **354**, 111–116 (2005).

29. Falcone, C. et al. The -374T/A RAGE polymorphism protects against future cardiac events in nondiabetic patients with coronary artery disease. *Arch. Med. Res.* **39**, 320–325 (2008).

30. Lindholm, E. et al. The -374 T/A polymorphism in the gene encoding RAGE is associated with diabetic nephropathy and retinopathy in type 1 diabetic patients. *Diabetologia* **49**, 2745–2755 (2006).

31. Ng, Z. X., Kuppusamy, U. R., Tajunisah, I., Fong, K. C. & Chua, K. H. Association analysis of −429T/C and −374T/A polymorphisms of receptor of advanced glycation end products (RAGE) gene in Malaysian with type 2 diabetic retinopathy. *Diabetes Res. Clin. Pract.* **95**, 372–377 (2012).

32. Wang, J. et al. Meta-analysis of RAGE gene polymorphism and coronary heart disease risk. *PLoS ONE* **7**, e50790 (2012).

33. Maillard-Lefebvre, H. et al. Soluble receptor for advanced glycation end products: a new biomarker in diagnosis and prognosis of chronic inflammatory diseases. *Rheumatology* **48**, 1190–1196 (2009).

34. Ballas, S. K. & Marcolina, M. J. Determinants of red cell survival and erythropoietic activity in patients with sickle cell anemia in the steady state. *Hemoglobin* **24**, 277–286 (2000).

35. Ballas, S. K., Kesen, M. R. & Goldberg M. F. Beyond the definitions of the phenotypic complications of sickle cell disease: an update on management. *ScientificWorldJournal* **2012**, 949535 (2012).

36. Vichinsky, E. et al. Comparison of organ dysfunction in transfused patients with SCD or β thalassemia. *Am. J. Hematol.* **80**, 70–74 (2005).

37. Sullivan, C. M. et al. RAGE polymorphisms and the heritability of insulin resistance: the Leeds family study. *Diabetes Vasc. Dis. Res.* **2**, 42–44 (2005).

38. Laki, J. et al. The HLA 8.1 ancestral haplotype is strongly linked to the C allele of −429T>C promoter polymorphism of receptor of the advanced glycation end-product (RAGE) gene. Haplotype-independent association of the −429C allele with high hemoglobinA1C levels in diabetic patients. *Mol. Immunol.* **44**, 648–655 (2007).

39. Peng, F. et al. Association of four genetic polymorphisms of AGER and its circulating forms with coronary artery disease: a meta-analysis. *PLoS ONE* **8**, e70834 (2013).

40. Peng, W. H. et al. RAGE gene polymorphisms are associated with circulating levels of endogenous secretory RAGE but not with coronary artery disease in Chinese patients with type 2 diabetes mellitus. *Arch. Med. Res.* **40**, 393–398 (2009).

41. Ramprasad, S. et al. RAGE gene promoter polymorphisms and diabetic retinopathy in a clinic-based population from South India. *Eye* **21**, 395 (2007).

42. Geroldi, D., Falcone, C. & Emanuele, E. Soluble receptor for advanced glycation end products: from disease marker to potential therapeutic target. *Curr. Med. Chem.* **13**, 1971–1980. (2006).

43. Balasubbu, S., Sundaresan, P. & Rajendran, A. Association analysis of nine candidate gene polymorphisms in Indian patients with type 2 diabetic retinopathy. *BMC Med. Genet.* **11**, article 158 (2010).

44. Rowisha, M., El-Batch, M., El Shikh, T., El Melegy, S. & Aly, H. Soluble receptor and gene polymorphism for AGE: relationship with obesity and cardiovascular risks. *Pediatr. Res.* **80**, 67 (2016).

45. Hudson, B. I., Stickland, M. H. & Grant, P. J. Identification of polymorphisms in the receptor for advanced glycation end products (RAGE) gene. *Diabetes* **47**, 1155 (1998).

46. Liu, L. & Xianc, K. RAGE Gly82Ser polymorphism diabetic microangiopathy. *Diabetes Care* **48**, 139 (1999).

47. Ahmed, S. G. & Ibrahim, U. A. A compendium of pathophysiologic basis of etiologic risk factors for painful vaso-occlusive crisis in sickle cell disease. *Niger. J. Basic Clin. Sci.* **14**, 57 (2017).

48. Kato, G. J., Steinberg, M. H. & Gladwin, M. T. Intravascular hemolysis and the pathophysiology of sickle cell disease. *J. Clin. Invest.* **127**, 750–760 (2017).