RESEARCH ARTICLE

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Exploration of Leucine-Rich Alpha-2 Glycoprotein 1 (LRG1) and Its Association with Proangiogenic Mediators in Sickle Cell Disease: A Potential Player in the Pathogenesis of the Disease

Orak Hücre Hastalığında Lösinden Zengin Alfa-2 Glikoprotein 1 (LRG1) ve Proanjiyogenik Mediyatörlerle İlişkisinin Araştırılması: Hastalığın Patogenezinde Potansiyel Bir Oyuncu

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Abstract

Objective: Leucine-rich alpha-2-glycoprotein 1 (LRG1) is a novel mediator involved in abnormal angiogenesis. We aimed to investigate circulating LRG1 levels and their relationship with proangiogenic mediators in sickle cell disease (SCD).

Materials and Methods: A total of 50 patients with SCD, with 25 in steady-state condition (SCD-SS) and 25 in periods of painful vaso-occlusive crisis (SCD-VOC), and 25 healthy controls were included in the study. Demographical and clinical data were collected from hospital records. Serum LRG1, vascular endothelial growth factor A (VEGFA), and hypoxia-inducible factor 1-alpha (HIF1A) levels were measured by enzyme-linked immunosorbent assay (ELISA), and C-reactive protein (CRP) was measured by the nephelometric method. Routine biochemical parameters were assessed using an autoanalyzer. Multinomial logistic regression was used to analyze ELISA parameters, and receiver operating characteristic (ROC) curves were constructed to determine the optimal cut-off point for HIF1A to predict VOCs in SCD patients.

Results: LRG1 and VEGFA levels were significantly higher in SCD patients than controls (p<0.001), with no difference between the SCD-SS and SCD-VOC groups. HIF1A, CRP, and lactate dehydrogenase levels differed significantly across all groups, being highest in the SCD-VOC group (p<0.001). After adjusting for age and sex, LRG1, HIF1A, and VEGFA remained elevated in the SCD groups. HIF1A correlated with CRP (r=0.351, p=0.024), but LRG1 showed no correlation with proangiogenic mediators in the SCD-VOC group. The area under the ROC curve was calculated as 0.694 (95% confidence interval: 0.542-0.845, p=0.021) and the optimal cut-off point was 494.5 pg/mL for HIF1A in predicting vaso-occlusive crises in patients with SCD.

Öz

Amaç: Lösinden zengin alfa-2-glikoprotein 1 (LRG1), anormal anjiyogenezde rol oynayan yeni bir mediyatördür. Bu çalışmada, dolaşımdaki LRG1 düzeylerini ve bu düzeylerin orak hücre hastalığında (OHH) proanjiyogenik mediyatörlerle ilişkisini araştırmayı amaçladık.

Gereç ve Yöntemler: Çalışmaya, 25'i stabil durumda (OHH-SS) ve 25'i ağrılı vazooklüzif kriz (OHH-VOK) döneminde olmak üzere toplam 50 OHH hastası ile 25 sağlıklı kontrol birey dahil edildi. Demografik ve klinik veriler hastane kayıtlarından elde edildi. Serum LRG1, vasküler endotelyal büyüme faktörü A (VEGFA) ve hipoksi ile indüklenebilir faktör 1-alfa (HIF1A) düzeyleri ELISA yöntemiyle, C-reaktif protein (CRP) düzeyleri ise nefelometrik yöntemle ölçüldü. Rutin biyokimyasal parametreler otoanalizör ile değerlendirildi. ELISA parametreleri çoklu lojistik regresyon analizi ile incelendi ve OHH hastalarında VOK'leri öngörmede HIF1A için optimal eşik değeri belirlemek amacıyla alıcı işletim karakteristik eğrisi (ROC) analizi yapıldı.

Bulgular: LRG1 ve VEGFA düzeyleri OHH hastalarında kontrol grubuna kıyasla anlamlı şekilde yüksek bulundu (p<0,001); ancak OHH-SS ve OHH-VOK grupları arasında anlamlı fark gözlenmedi. HIF1A, CRP ve laktat dehidrogenaz düzeyleri tüm gruplar arasında anlamlı farklılık gösterdi ve en yüksek düzeyler OHH-VOK grubunda saptandı (p<0,001). Yaş ve cinsiyete göre düzeltme yapıldıktan sonra LRG1, HIF1A ve VEGFA düzeylerinin OHH gruplarında yüksek seyretmeye devam ettiği görüldü. HIF1A düzeyi CRP ile pozitif korelasyon gösterdi (r=0,351, p=0,024); ancak OHH-VOK grubunda LRG1 düzeyi ile proanjiyogenik mediyatörler arasında bir korelasyon saptanmadı. ROC eğrisi altında kalan alan 0.694 olarak hesaplandı (güven aralığı, %95: 0,542-0,845, p=0,021) ve HIF1A için OHH hastalarında VOK'leri öngörmede optimal kesim noktası 494,5 pg/mL olarak belirlendi.

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Abstract

Conclusion: Circulating LRG1 levels may reflect neutrophil activation and contribute to the cross-talk between proangiogenic mediators released in SCD.

Keywords: Sickle cell disease, LRG1, Angiogenesis, VEGF, HIF1A

Öz

Sonuç: Dolaşımdaki artmış LRG1 düzeyleri nötrofil aktivasyonunu yansıtabilir ve OHH'de salınan proanjiyogenik mediyatörler arasındaki etkileşime katkı sağlayabilir.

Anahtar Sözcükler: Orak hücre hastalığı, LRG1, Anjiyogenez, VEGFA, HIF1A

Introduction

Sickle cell disease (SCD), constituting a group of autosomal recessive hemoglobinopathies, are prevalent in the Çukurova region of Türkiye, and particularly in Hatay province [1]. It is a monogenetic disorder caused by a single base-pair mutation in the *β-globin* gene. This mutation results in the substitution of valine for glutamic acid at the 6th position of the β chain, forming abnormal hemoglobin (HbS) [2,3]. The polymerization of deoxygenated HbS results in the transformation of red blood cells from their biconcave shape into a sickle cell shape under low-oxygen conditions, leading to vaso-occlusion, especially in small vessels. Continuous and intermittent microvascular blockages initiate complex pathophysiological mechanisms, encompassing ischemia/reperfusion injury, hypercoagulability, heightened leukocyte adhesion to the endothelium, and ultimately tissue damage [4].

While the ischemic events of SCD have been extensively investigated, the role of hypoxia-induced angiogenic responses has not been fully elucidated in patients with SCD. Hypoxia-inducible factor 1-alpha (HIF1A) plays a pivotal role in maintaining oxygen homeostasis and is upregulated during hypoxia [5]. Under hypoxic or ischemic conditions, HIF1A initiates the upregulation of gene transcription and associated mediators responsible for promoting angiogenesis, such as vascular endothelial growth factor A (VEGFA). There are limited studies showing increased HIF1A expression levels in SCD [6,7]. Elevated levels of VEGFA have also been observed in patients with SCD, attributed to factors including hypoxia, ischemia, and vascular damage [7,8,9]. However, some studies have reported inconsistent findings [10]. While these observations suggest a correlation between hypoxia and angiogenesis, the presence of proangiogenic mechanisms in SCD remains unclear. Among the emerging factors, leucine-rich alpha-2-glycoprotein 1 (LRG1) is a secreted glycoprotein belonging to the leucine-rich repeat protein family [11]. LRG1 is primarily secreted from neutrophils, but it is also released from other cell types, including endothelial cells, epithelial cells, and fibroblasts [12]. Its primary function involves modulating angiogenesis, specifically pathological angiogenesis, by promoting vascular remodeling through the transforming growth factor beta (TGF-β) signaling pathway [13]. Elevated levels of LRG1 have been documented in diseases characterized by abnormal angiogenesis, such as diabetic kidney disease [14], multiple myeloma [15], and osteoarthritis [16].

However, there have been no studies investigating LRG1 levels in SCD. Considering the unbalanced angiogenesis resulting from hypoxia in patients with SCD, investigating the role of LRG1 may contribute to the elucidation of the cross-talk between angiogenetic and inflammatory mediators, including VEGF and HIF1A.

This is the first study evaluating serum LRG1 levels in patients with SCD. The aim of this study is to examine serum LRG1 levels and their relationships with VEGFA, HIF1A, and acute-phase reactants in patients with SCD during steady-state (SS) conditions and painful vaso-occlusive crisis (VOC) episodes.

Materials and Methods

Study Design

This was a prospective case-control study. A total of 50 patients with SCD as proven by Hb electrophoresis (homozygous sickle cell disease [HbSS] or sickle cell beta thalassemia [HbS/β-thal]) presenting to the Hematology Department of Hatay Mustafa Kemal University Hospital (Hatay, Türkiye) between December 2021 and January 2023 were included in the study. The control group consisted of 25 age- and sex-matched healthy subjects. Data on all relevant demographic and clinical features were obtained from the hospital's information system and recorded. Of the 50 patients with SCD, 25 were in SS condition, constituting the SCD-SS group, and 25 were in a period of painful VOC, constituting the SCD-VOC group. An acute VOC phase was clinically defined as a painful episode lasting more than 2 hours with the patient feeling that the pain was specific to vaso-occlusion. Doctors could not identify another etiology for the pain in these cases and the patients sought treatment for pain in the emergency department. The patients in the SS period were clinically defined as patients who had not been in VOC for at least 1 month before study inclusion [17].

Informed written consent was obtained from all patients and healthy control participants. This study was performed according to the Declaration of Helsinki's ethical principles for human medical research and the study protocol was approved by the Hatay Mustafa Kemal University Ethics Committee (protocol date and number: 06.05.2021 and 2021/47, decision no: 11).

Exclusion Criteria

Individuals with any hematological disorders other than SCD and patients with chronic pain unrelated to SCD were excluded.

Patients who had acute chest syndrome or other SCD-related complications within the past year as well as those with chronic kidney disease, inflammatory or connective tissue disease, acute or chronic infections, diabetes, obesity, liver failure, or coronary heart disease were excluded, as were smokers, alcohol consumers, those who had received transfusions in the last 3 months, individuals under 18 years of age, pregnant women, and breastfeeding women.

Sample Collection and Measurement of Biochemical Parameters

Whole blood (8 mL) for clot-activator gel tubes (BD Vacutainer Serum Separation Tubes, Becton Dickinson, Franklin Lakes, NJ, USA) and samples of blood with EDTA (4 mL) (BD Vacutainer K_2EDTA tubes, Becton Dickinson) were collected from SCD patients and the members of the healthy control group. In the SCD-VOC group, blood samples were collected within the first hour of presentation before administering any medication to the patient. After centrifugation at 1500 x g for 10 min (NF 1200 centrifuge, Nüve, Akyurt, Türkiye), samples were aliquoted for the measurement of routine biochemical data and enzyme-linked immunosorbent assay (ELISA) analyses and stored at -80 °C. Serum albumin, blood urea nitrogen, and creatinine levels and alanine aminotransferase, aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) activities were measured by the spectrophotometric method using an autoanalyzer (Advia 2400, Siemens, Tokyo, Japan), and serum high-sensitivity C-reactive protein (CRP) levels were measured by the nephelometric method (Advia 1200, Siemens). Hematological parameters were assayed using a whole blood count analyzer (Mindray BC-6800, Mindray, Shenzhen, China) within 2 hours of collection.

The serum LRG1, HIF1A, and VEGFA levels of all samples were measured by the ELISA method using commercially available ELISA kits (Elabscience, Wuhan, China) according to the manufacturer's protocol for the ELISA device (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, USA). Concentrations were calculated using a 4P-logic calibration curve, and the performance characteristics of the kits were as follows:

LRG1 ELSIA Kit (LOT: E-EL-H1287): Analysis range of 7.81-500 ng/mL, with sensitivity of 4.69 ng/mL; intra-assay coefficient of variability (CV) of <8% and inter-assay CV of <10%; final concentrations calculated by multiplying the dilution factor and given as µg/mL.

HIF1A ELISA Lit (LOT: E-EL-H6066): Analysis range of 62.5-4000 pg/mL, with sensitivity of 37.5 pg/mL; intra-assay CV of <8% and inter-assay CV of <10%.

VEGFA ELISA Kit (LOT: E-EL-H0111): Analysis range of 31.25-2000 pg/mL, with sensitivity of 18.75 pg/mL; intra-assay CV of <8% and inter-assay CV of <10%.

Statistical Analysis

IBM SPSS Statistics for Windows 21.0 (IBM Corp., Armonk, NY, USA) was used to analyze the data. Power analysis was performed using an effect size (Cohen's f) of 0.44, with alpha level of 0.05 and power of 0.80. The effect size was determined based on similar studies in the literature and pilot data previously obtained. The required sample size for each group was calculated to be at least 18 individuals. The normality of the data was evaluated using the Shapiro-Wilk test. Normally distributed variables were presented as mean ± standard deviation, whereas non-normally distributed variables were characterized by median (25^{th}-75^{th} interquartile range) values. Categorical data were analyzed using the chi-square test. Continuous variables with normal distribution were analyzed by one-way analysis of variance (ANOVA), followed by post-hoc Tukey or Tamhane T2 tests as appropriate. For non-normally distributed continuous variables, the Kruskal-Wallis test with post-hoc Bonferroni multiple comparison testing was utilized. The relationships between variables were assessed using the Pearson correlation test for normally distributed data and the Spearman correlation test for non-normally distributed data. Multinomial logistic regression analysis was conducted to evaluate the relationship between SCD groups and ELISA parameters. In the regression model, the ELISA parameters were defined as independent variables, while the disease groups (SCD-SS and SCD-VOC) were defined as dependent variables, with the control group serving as the reference category. Adjustments were made for age and sex, and the resulting odds ratios and 95% confidence intervals (CIs) were reported. We also constructed receiver operating characteristic (ROC) curves and calculated the sensitivities and specificities of HIF1A. The optimal cut-off point for HIF1A was detected for the prediction of VOC in patients with SCD. Values of $p<0.05$ were considered statistically significant.

Results

The demographic distribution and laboratory results of the participants are presented in Table 1. Of the 50 patients with SCD, 25 (50%) were male and 25 (50%) were female, with a mean age of 34±9 years. There were no statistically significant differences in age or sex between any of the three groups. The majority of the patients (n=47, 94%) had HbSS, while only 3 (6%) had sickle/β-thalassemia (HbS/β-thal). Fifteen patients (60%) in the SCD-VOC group and 13 patients (52%) in the SCD-SS group were using hydroxyurea.

Serum CRP levels and LDH activities were significantly different between all three groups, being highest in the SCD-VOC group ($p<0.001$). White blood cell (WBC) and neutrophil counts and AST activities were found to be higher in SCD patients compared to the healthy controls ($p<0.001$), but there was no significant difference between the SCD-VOC and SCD-SS groups. Hb

levels were significantly different between all three groups, being lowest in the SCD-VOC group, but albumin levels were significantly lower only in the SCD-VOC group (p<0.001) (Table 1). Serum LRG1 levels were significantly higher in both the SCD-SS and SCD-VOC groups compared to the control group (p=0.003 and p=0.001, respectively). There was no significant difference observed in serum LRG1 levels between the SCD-SS and SCD-VOC groups (Figure 1A). Serum HIF1A levels were significantly higher in both the SCD-SS and SCD-VOC groups compared to the controls (p<0.001) (Figure 1B), and the highest levels were observed in the SCD-VOC group (p=0.006). VEGFA levels were significantly higher in both the SCD-SS and SCD-VOC groups compared to the control group (p=0.004 and p<0.001, respectively), with no significant difference observed between the SCD-SS and SCD-VOC groups (Figure 1C). In the VOC-SCD group, serum HIF1A levels positively correlated with CRP levels (r=0.351, p=0.024). There were no significant correlations observed between LRG1 and VEGFA (r=0.047, p=0.832) or LRG1 and HIF1A (r=0.085, p=0.699) levels in the SCD-VOC and SCD-SS groups. According to the multinomial logistic regression analysis, after adjusting for age and sex, the SCD-SS and SCD-VOC groups had significantly higher levels of LRG1, HIF1A, and VEGFA compared to the control group (Table 2). The area under the curve (AUC) was calculated as 0.694 (95% CI: 0.542-0.845, p=0.021) and the optimal cut-off point was 494.5 pg/mL for HIF1A in predicting VOC in patients with SCD (Figure 2). The HbS levels of the SCD-SS and SCD-VOC groups were 76.5% (58.6%-88.4%) and 79.5% (76.5%-84.4%), respectively, with no significant difference between the groups (p=0.572). HbS levels were not correlated with LRG1, HIF1A, or VEGFA levels in SCD patients (r=0.099 and p=0.748, r=0.421 and p=0.152, and r=-0.022 and p=0.940, respectively).

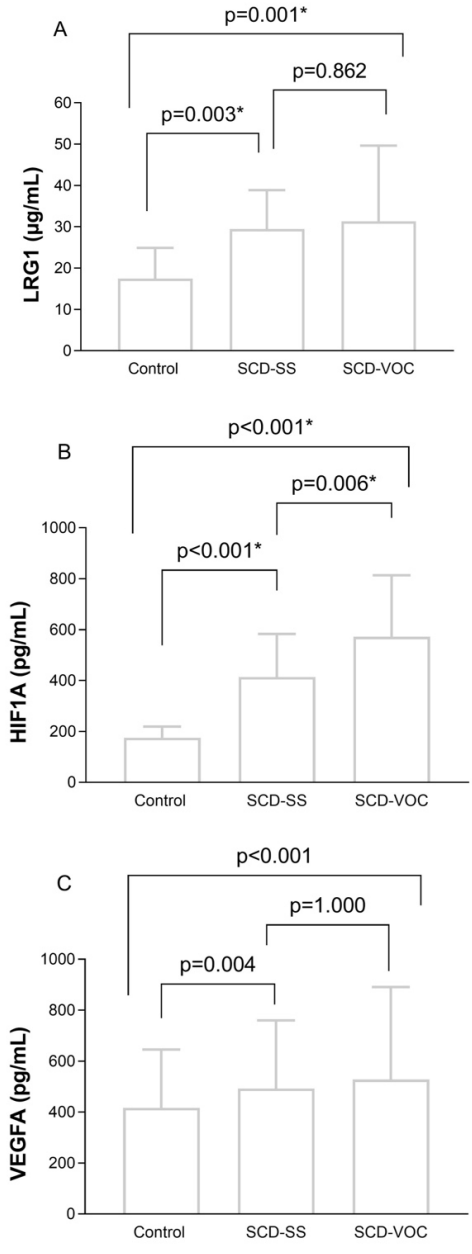

Figure 1. Comparisons of serum LRG1 (A), HIF1A (B), and VEGFA (C) among the groups.

*: ANOVA testing was used for normally distributed parameters. Kruskal-Wallis tests were conducted for other parameters. SCD-SS: Patients with sickle cell disease in steady-state condition; SCD-VOC: patients with sickle cell disease in painful periods of vaso-occlusive crisis; LRG1: leucine-rich alpha-2-glycoprotein 1; HIF1A: hypoxia-inducible factor 1-alpha; VEGFA: vascular endothelial growth factor A.

Discussion

In this study, serum levels of LRG1 and proangiogenic mediators HIF1A and VEGFA were measured at different stages of SCD.

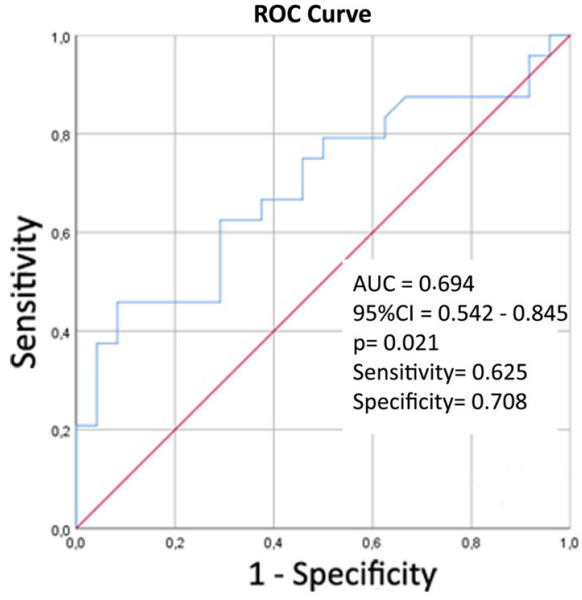

Figure 2. Receiver-operating characteristic (ROC) curve to determine the optimal threshold value for HIF1A in patients with SCD. The optimal cut-off point was 494.5 pg/mL for HIF1A parameter to estimate VOC in patients with SCD.

HIF1A: Hypoxia-inducible factor 1-alpha; SCD: sickle cell disease; VOC: vaso-occlusive crisis; AUC: area under the curve; CI: confidence interval.

Both LRG1 and VEGFA levels were significantly elevated in SCD patients compared to the healthy control group, with no significant differences observed between SS and VOC periods, which displayed similar patterns. Serum HIF1A levels were also significantly higher in SCD patients, with the highest levels observed in the VOC group, in contrast to LRG1 and VEGFA. Additionally, a significant but low correlation was observed between serum HIF1A and CRP levels, while no correlation was found between LRG1 and the proangiogenic mediators. HIF1A demonstrated better ability to distinguish VOC and SS conditions in SCD patients.

LRG1 levels were significantly elevated in both the SCD-SS and SCD-VOC groups compared to the healthy control group in this study (p=0.003 and p=0.001, respectively) (Figure 1A; Table 1).

Table 1. Comparisons of age, sex, and levels of biochemical parameters among the study groups.

Variables	Control (n=25) Mean ± SD Median (25^{th}-75^{th})	SCD-SS (n=25) Mean ± SD Median (25^{th}-75^{th})	SCD-VOC (n=25) Mean ± SD Median (25^{th}-75^{th})	p
Female, n (%)	17 (68)	11 (44)	14 (56)	0.232*
Age, years	33±7	35±9	33±9	0.715**
WBC, 10^3/µL	7.2 (5.9-8.3)	11.4 (6.9-13.7)	13.4 (10.7-17.6)	0.014[a] <0.001[b] 0.164[c]
Neutrophil counts, 10^3/µL	4.1 (3.4-5.2)	6.1 (4.1-8.9)	9.3 (6.3-13.1)	0.024[a] <0.00[b] 0.145[c]
Hemoglobin, g/dL**	14±1.6	9.3±2.1	7.7±1.1	<0.001[a] <0.001[b] 0.002[c]
Platelets, 10^3/µL	243 (211-288)	361 (219-444)	317 (184-415)	0.065
hsCRP, mg/L	0.92 (0.64-1.71)	4.39 (3.13-7.73)	34.7 (10.5-61.5)	<0.001[a] <0.001[b] 0.003[c]
Albumin, g/dL	4.6 (4.4-4.7)	4.3 (4.2-4.7)	3.7 (3.6-3.9)	0.398[a] <0.001[b] <0.001[c]
ALT, U/L	17 (14-26)	18 (14-29)	25 (14-33)	0.416
AST, U/L	15 (13-20)	40 (27-62)	50 (36-60)	<0.001[a] <0.001[b] 0.791[c]
LDH, U/L	145 (89-201)	339 (238-542)	638 (491-680)	<0.001[a] <0.001[b] 0.016[c]
BUN, mg/dL	11 (10-14)	9 (8-13)	8 (6-11)	0.280[a] 0.252[b] 0.002[c]
Creatinine, mg/dL	0.69 (0.61-0.79)	0.49 (0.42-0.61)	0.42 (0.34-0.55)	<0.001[a] <0.001[b] 0.813[c]
LRG1 (µg/mL)**	17.5±7.5	29.5±9.4	31.3±18.3	0.003[a] 0.001[b] 0.862[c]
HIF1A (pg/mL)**	174.8±44.7	413.5±169.3	572.1±241.5	<0.001[a] <0.001[b] 0.006[c]
VEGFA (pg/mL)	416 (374-484)	492 (417.5-630.5)	528 (446.5-589)	0.004[a] <0.001[b] 1.000[c]

*: Chi-square test; **: analysis of variance (ANOVA). Kruskal-Wallis tests were conducted for other parameters. [a]: Control group vs. SCD-SS group; [b]: Control group vs. SCD-VOC group; [c]: SCD-SS group vs. SCD-VOC group.

SCD-SS: Patients with sickle cell disease in steady-state condition; SCD-VOC: patients with sickle cell disease in painful periods of vaso-occlusive crisis. WBC: White blood cells; hsCRP: high-sensitivity C-reactive protein; ALT: alanine aminotransferase; AST: aspartate aminotransferase; LDH: lactate dehydrogenase; BUN: blood urea nitrogen; LRG1: leucine-rich alpha-2-glycoprotein 1; HIF1A: hypoxia-inducible factor 1-alpha; VEGFA: vascular endothelial growth factor A; SD: standard deviation.

Table 2. Multinomial logistic regression for the association between study groups and ELISA parameters.

		LRG1	HIF1A	VEGFA
Control		1 (reference)	1 (reference)	1 (reference)
SCD-SS	OR (95% CI)*	1.15 (1.053-1.256)	1.025 (1.010-1.042)	1.014 (1.005-1.022)
	p	0.002	0.002	0.002
SCD-VOC	OR (95% CI)*	1.2 (1.076-1.339)	1.029 (1.013-1.046)	1.015 (1.007-1.024)
	p	0.001	<0.001	<0.001

*: Adjusted for age and sex. The dependent variables were the control, SCD-SS, and SCD-VOC groups as categorical groups, with the control group serving as the reference. The presence of multicollinearity among independent variables was assessed using variance inflation factors (VIFs), and no variable with VIF of >5 was detected. Odds ratios (ORs) and their corresponding 95% confidence intervals (CIs) are presented.
SCD-SS: Patients with sickle cell disease in steady-state condition; SCD-VOC: patients with sickle cell disease in painful periods of vaso-occlusive crisis; LRG1: leucine-rich alpha-2-glycoprotein 1; HIF1A: hypoxia-inducible factor 1-alpha; VEGFA: vascular endothelial growth factor A.

Additionally, serum LRG1 levels remained significantly higher in the SCD-SS and SCD-VOC groups in multivariable models after adjusting for age and sex (Table 2). However, there was no significant difference between the VOC and SS periods. The elevated levels of LRG1 in SCD patients could possibly be associated with its secretion kinetics because LRG1 is packaged into the granule compartment of human neutrophils and secreted upon neutrophil activation to modulate the microenvironment [18]. Considering the increased granulopoiesis in SCD patients [19,20,21], we can say that LRG1 may be upregulated by the chronic systemic inflammation observed in SCD patients and contribute to microcirculatory irregularities. The significantly elevated WBC and neutrophil counts in both the SCD-VOC and SCD-SS groups compared to the control group in this study further support this idea (Table 1).

We also found higher serum CRP levels in patients compared to the control group, but its levels were highest in the SCD-VOC group (Table 1). CRP is a well-known acute-phase reactant and a laboratory indicator of the severity of inflammation in SCD. However, in the VOC group, there was no significant correlation between LRG1 levels and CRP, WBC, or neutrophil counts. Levels of LRG1 did not vary with disease severity. We can say that LRG1 is not an effective biomarker for distinguishing between crisis and remission periods in cases of SCD. Therefore, further studies are needed to understand the role of LRG1 as an acute-phase protein in patients with SCD. However, the significantly elevated levels of circulating LRG1 in patient groups suggest its potential involvement in the angiogenic imbalance associated with SCD. LRG1 exerts these effects by activating the proangiogenic pathway, promoting endothelial cell proliferation, migration, and tubulogenesis [11]. It has also been reported that LRG1 enhances TGF-β signaling in the endothelium, contributing to abnormal vascular growth in inflammatory and ischemic conditions [13]. This mechanism may also play a role in the pathogenesis of SCD. In a study conducted on a cardiomyocyte cell line, it was demonstrated that LRG1 significantly enhanced the expression of HIF1A [22]. Another study reported that LRG1 induces HIF1A and regulates epithelial-mesenchymal transition and angiogenesis in colorectal cancer [23]. Moreover, LRG1 has been demonstrated to guide glomerular endothelial cells toward a proangiogenic pathway [24] and to enhance ocular neovascularization in diabetic retinopathy [13]. In the present study, serum levels of VEGFA and HIF1A as proangiogenic mediators were also measured. Serum HIF1A levels were significantly increased in both the SCD-SS and SCD-VOC groups compared to healthy controls (p<0.001) (Figure 1B). This difference was significant after adjustment for age and sex (Table 2). HIF1A is a widely recognized key transcription factor that becomes activated in response to hypoxia and plays a vital role in restoring oxygen homeostasis [25,26]. Increased expression of HIF1A in SCD patients has been demonstrated in a limited number of studies [7,27]. Our findings are consistent with those of previous studies and support the idea of elevated HIF1A levels in SCD patients. Unlike LRG1, its levels were significantly higher in the SCD-VOC group compared to the SCD-SS group (p=0.006) (Figure 1B) and correlated positively with CRP levels (r=0.351, p=0.024). This was an anticipated result, as elevated levels of HIF1A in the SCD-VOC group may be associated with the severe hypoxia experienced by these patients. We also performed ROC analysis to determine the optimal threshold value for HIF1A in patients with SCD. The AUC was calculated as 0.694 (95% CI: 0.542-0.845, p=0.021) (Figure 2). Thus, we can say that a serum HIF1A cut-off value of 494.5 pg/mL is feasible for predicting VOC in SCD patients.

The elevated HIF1A levels observed in patients during the SS period were likely a result of ongoing chronic inflammation, as suggested by a previous study [28]. During hypoxia, it has been demonstrated that HIF1A rapidly binds to the regulatory region of the VEGFA-expressing gene, thereby initiating its transcription and translation [29,30]. In the present study, VEGFA levels were also found to be elevated in patients during the SS and VOC periods compared to the controls (p=0.004 and p<0.001, respectively), similar to LRG1. However, no significant difference was observed between the two periods (Figure 1C). VEGFA has been previously investigated in patients with SCD; however, conflicting results have been reported. While some

studies have found no differences in VEGFA levels between patients with SCD during VOC and SS periods [10,31], others have reported higher VEGFA levels during VOC episodes [32]. The differences between studies could be related to the antiangiogenic effects of hydroxyurea, a drug used in SCD treatment. One study suggested that hydroxyurea reduces VEGFA and HIF1A expression under both in vivo and in vitro conditions [33]. Although the underlying mechanism has not yet been elucidated, hydroxyurea treatment may affect serum LRG1 and VEGF levels through novel vascular mechanisms in the patients at different stages of SCD. The use of hydroxyurea was comparable between the two patient groups during the SS (52%) and VOC (60%) periods in the present study. This may explain the lack of a significant difference between the two groups. Serum VEGFA levels demonstrated a pattern similar to that of serum LRG1 in patients with SCD in this study. A similar relationship was reported in another study conducted on tumor cells, wherein LRG1 was shown to directly induce VEGFA expression and promote angiogenesis in colorectal cancer cells [23]. Another study showed that knockdown of LRG1 dramatically reduced VEGFA expression in the mouse retina [13]. In the present study, the elevated LRG1 and VEGFA levels of SCD patients may have caused the formation of non-functional vessels, contributing to the unbalanced angiogenesis triggered by ischemic processes.

Study Limitations

The first limitation of this study is its small sample size due to its single-center design. Another limitation is drug use. More than half of the patients were receiving hydroxyurea treatment, which has previously been reported to affect the serum levels of some markers. Therefore, further studies with larger case groups considering drug usage are needed.

Conclusion

In this study, we observed elevated levels of LRG1 in SCD patients together with VEGF and HIF1A as other proangiogenic mediators. HIF1A may prove to be more useful in distinguishing between VOC and SS periods of SCD. Circulating LRG1 levels may reflect neutrophil activation in SCD and contribute to the cross-talk between proangiogenic mediators released during hypoxia. However, the small cohort of this study does not allow the definitive confirmation of LRG1 as a biomarker for VOC. Larger studies with more extensive sample sizes are needed to validate its potential as a biomarker in patients with SCD during the VOC period.

Ethics

Ethics Committee Approval: This study was performed according to the Declaration of Helsinki's ethical principles for human medical research and the study protocol was approved by the Hatay Mustafa Kemal University Ethics Committee (protocol date and number: 06.05.2021 and 2021/47, decision no: 11).

Informed Consent: Informed written consent was obtained from all patients and healthy control participants.

Footnotes

Authorship Contributions

Surgial and Medical Practices: M.K., L.S.D.B.; Concept: O.Ö., M.K.; Design: O.Ö., M.K.; Data Collection or Processing: F.H.E., H.O., L.S.D.B.; Analysis or Interpretation: O.Ö., F.H.E., H.O.; Literature Search: O.Ö. F.H.E., L.S.D.B.; Writing: O.Ö., M.K., H.O. H.K., A.A.

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