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SEVERE SICKLE CELL ANEMIA IS ASSOCIATED WITH INCREASED PLASMA LEVELS OF TNF-R1 AND VCAM-1

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Abstract

Sickle cell anemia (SCA, *HBB* glu6val) is characterized by multiple complications and a high degree of phenotypic variability: some subjects have only sporadic pain crises and few acute hospitalizations, while others experience multiple serious complications, high levels of morbidity, and accelerated mortality. ¹ The tumor necrosis factor- α (TNF- α) signaling pathway plays important roles in inflammation and the immune response; variation in this pathway might be expected to modify the overall severity of SCA through the pathway's effects on the vascular endothelium.^{2, 3} We examined plasma biomarkers of TNF- α activity and endothelial cell activation for associations with SCA severity in 24 adults (12 mild, 12 severe). Two biomarkers, tumor necrosis factor- α receptor-1 (TNF-R1) and vascular cell adhesion molecule-1 (VCAM-1) were significantly higher in subjects with severe SCA. Along with these biomarker differences, we also examined data from a genome-wide association study (GWAS) using SCA severity as a disease phenotype, and found evidence of genetic association between disease severity and a single nucleotide polymorphism (SNP) in *VCAM1*, which codes for VCAM-1, and several SNPs in *ARFGEF2*, a gene involved in TNF-R1 release.⁴

Keywords

Sickle Cell Anemia; TNF- α ; Disease Severity

Exposure to TNF- α activates endothelial cells, leading to up-regulation and enhanced cell-surface expression of multiple proteins including VCAM-1 and intra-cellular adhesion molecule-1 (ICAM-1).⁵ During acute infections, these proteins facilitate the attachment of leukocytes to the vessel wall, ultimately resulting in transmigration into infected tissue.⁶ In SCA, variable TNF- α signaling might alter disease severity by activating systemic and pulmonary endothelium and by modulating rates of sickle vasoocclusion secondary to altered leukocyte-endothelial cell adhesion.^{7, 8} Associations between some individual clinical complications of SCA and plasma levels of VCAM-1 and ICAM-1, but not TNF-R1, have been described previously.^{9, 10} Here, we report associations between increase plasma levels of VCAM-1 and TNF-R1 and severe SCA, as measured by the SCA severity score, a validated metric which incorporates information about steady-state laboratory data and

medical history into a Bayesian network in order to estimate a subject's probability of dying within the next five years due to complications of SCA.^{11, 12}

In a population of 52 adult subjects with SCA recruited for a study of pulmonary hypertension, we used the severity score to identify the 12 subjects with the most mild clinical presentations (probability of death within five years = $7.5 \pm 2.6\%$, mean $\pm$ sd.) and the 12 subjects with the most severe presentations (probability = $80.3 \pm 18\%$). No significant differences were observed between the two groups in terms of age or hydroxyurea treatment (p-values 0.35 and 0.68, respectively), while non-significant trends toward being female and having lower glomerular filtration rates (GFR) were observed in the severe group (p-values 0.1, 0.1). Subjects in the severe group had lower levels of hemoglobin than subjects in the mild group (median 8.3 vs. 9.45 g/dL, p-value = 0.046), though the difference was not significant after adjusting for multiple testing.

To look for associations between SCA severity and TNF- α axis activity and endothelial cell activation, steady-state plasma samples from these two groups were tested for levels of E-selectin, ICAM-1, nitric oxide (NO), P-selectin, TNF- α , TNF-R1, and VCAM-1. Only TNF-R1 and VCAM-1 showed significant differences between the two groups after correcting for multiple testing; both biomarkers were significantly higher in severe cases of SCA (p-value = 0.0015 and p-value = 0.0019, respectively, Table 1). Plasma levels of TNF-R1 and VCAM-1 showed a significant correlation after log transformation (Figure 1, p-value = 0.036).

Renal disease, a common occurrence in SCA, can lead to increased plasma levels of TNF-R1 secondary to decreased renal clearance.^{13, 14} To evaluate this and other possible confounding factors, we tested for associations between TNF-R1 or VCAM-1 levels and GFR, gender, age, and hydroxyurea treatment status. We also looked for potential association with plasma nitric oxide (NO), since endothelial cell-surface expression of leukocyte adhesion proteins is altered by NO bioavailability.^{15, 16, 17} TNF-R1 levels were found to be associated with gender (p-value = 0.048), but were not significantly associated with GFR, hydroxyurea use, NO levels, or with age (p-value > 0.05 in all cases). VCAM-1 levels were not significantly associated with any of these factors (p-value > 0.05). The relationship between TNF-R1 and SCA severity remained significant in a linear regression model involving both TNF-R1 and gender (p-value = 4.6×10^{-03}), indicating that TNF-R1 as a plasma biomarker is significantly related to increased SCA severity beyond its association with gender. In contrast to our results, multiple groups have shown that hydroxyurea therapy was associated with decreased levels of multiple inflammatory biomarkers, including VCAM-1; this may be due to our small sample size, or to differences in efficacy of hydroxyurea treatment.^{9, 18, 19}

We identified 155 SNPs in 16 candidate genes which either code for one of the six biomarkers, or are involved in their release into the plasma. SNP data was obtained from a previous GWAS, which examined 1265 subjects with SCA from the Cooperative Study of Sickle Cell Disease (CSSCD) using the severity score as a phenotype. While none of the 155 SNPs met stringent criteria for genome-wide significance, several of these SNPs did show significant association with SCA severity at moderate levels (BF > 3; Table 2).⁴

Of the 47 SNPs examined in the six candidate genes coding for the plasma biomarkers (*ICAM1*, *SELE*, *SELP*, *TNF*, *TNFRSF1A*, and *VCAM1*), only one (rs1041163), located in the promoter of *VCAM1*, was significantly associated with SCA severity (BF = 3.5). This same SNP was previously reported to be associated with small vessel stroke in the CSSCD.²⁰ We also examined 108 SNPs in 10 genes involved in the release of these biomarkers or in the generation of NO (*ADAM17*, *ARFGEF2*, *ARF1*, *ARF3*, *ERAP-1*, *IL1B*, *NOS1*, *NOS2A*,

NOS3, and *NUCB2*).^{21–25} Of these SNPs, the top association was with rs2273102, located in intron 12 of *ARFGEF2*, a gene known to be involved in endothelial release of TNF-R1 into circulation in an *ADAM17*-independent manner (BF = 70 under a dominant model).²⁴ Three of the other 15 SNPs in *ARFGEF2* (rs6019548, rs1115535, and rs6019566) also showed significant associations with SCA severity (BF > 3). Using SNP set enrichment (SSE) analysis, which summarizes genetic association across multiple SNPs within a single gene, *ARFGEF2* as a whole was found to be significantly related to SCA severity (p-value = 5.5×10^{-05}).⁴ Significant association with SCA severity was also observed with rs1143634, located in the gene *IL1B* (BF > 30 under a recessive model). *IL1B* codes for the cytokine IL-1B, which induces *ADAM17*-dependent release of TNF-R1 into circulation, among other roles.²¹ None of the other 15 genes were significantly related to SCA severity by SSE analysis.

Our data showing severity-related variability in plasma levels of biomarkers of TNF- α signaling, together with genetic association between SCA severity and genes involved in biomarker generation and release, supports the hypothesis that genetic variability along the TNF- α signaling pathway may modulate the severity of SCA. Further investigation, including the confirmation of both the genetic and biomarker associations with SCA severity in a single population, is necessary to develop a more complete understanding of the ways in which genetic variation in TNF- α signaling might affect the clinical manifestations of this disease. With such confirmation, the associations shown here between SCA severity and variability along the TNF- α signaling pathway might be used to further prospectively stratify subjects with SCA into different levels of risk, and, ultimately, may lead to the ability to more rationally designed therapeutic plans for subjects with different clinical presentations of SCA.

METHODS

Study populations

Subjects included in the biomarker analysis were recruited as part of a study conducted at Boston Medical Center (BMC) to identify genetic modifiers associated with pulmonary hypertension in sickle cell disease.¹⁰ Peripheral blood samples were collected when the subjects were at least two weeks removed from a vasoocclusive crisis, and at least four weeks removed from an episode of acute chest syndrome. Platelet-poor plasma samples were prepared as described previously and stored at -70°C until the time of the study.¹⁰ Of the 52 subjects in the BMC group available at the time of this study, the 24 with the most extreme severity scores were selected for complete biomarker analysis. For these 24 subjects, GFR was estimated using the Modification of Diet in Renal Disease formula adjusted for use in African-Americans.²⁶ In one case, GFR estimation was not possible as a creatinine level was not recorded at the time of sample collection. Subjects included in the genetic association study were described fully in a previous report.⁴ Briefly, the genetic association population set was comprised of 1265 subjects with SCA (1088 mild and 177 severe), originally recruited as part of the Cooperative Study of Sickle Cell Disease (CSSCD).²⁷ These studies were approved by the Institutional Review Board at Boston Medical Center.

Severity score calculation

Severity score was calculated using a Bayesian network described previously, and available for download at <http://www.bu.edu/sicklecell/downloads/Projects>.¹¹ For subjects in the biomarker analysis, severity score was calculated at study enrollment, coincident with phlebotomy for plasma samples. For subjects in the CSSCD, severity scores were calculated

using the median values of data acquired over multiple longitudinal visits, with thresholds for defining severe cases of SCA described previously.^{11, 14}

Quantification of plasma biomarker levels

TNF- α , TNF-R1, and P-selectin were assayed by enzyme-linked immunoassay (ELISA) using commercially available kits from R & D Biosystems (Minneapolis, MN). VCAM-1, ICAM-1, and E-selectin levels were assayed by ELISA using kits from Invitrogen (Carlsbad, CA). Nitric oxide metabolites were assayed as nitrate/nitrite levels using a colorimetric assay (Cayman Chemical, Ann Arbor, MI).

Statistical Analysis

Univariate associations between individual biomarker levels and SCA severity, gender, or hydroxyurea therapy status were examined using Mann-Whitney U tests. Correlations between biomarker levels and age or estimated GFR were examined using Spearman correlation tests and Pearson correlation for log-transformed values. An overall significance level of 0.05 was set as significant for all univariate tests, and a Bonferonni correction was used to adjust for multiple testing (Bonferonni corrected p-value = 0.0029). For multivariate analysis, biomarker levels were natural-log-transformed and fit to linear models involving SCA severity with or without other variables, as described in the text. All univariate tests and linear models were performed in R (available at www.r-project.org).

Genetic association study and SNP-Set Enrichment (SSE) Score

DNA isolated from peripheral blood samples from the 1265 subjects in the genetic association population was analyzed for >600k SNPs using the Illumina Human 610-Quad chip, according to the manufacturer's protocol. Potential associations between SCA severity and individual SNPs were tested using Bayesian methods described in detail elsewhere.^{28, 29} SNPs with Bayes Factor (BF) > 3 under any model were considered to show evidence of association with SCA severity with a false positive rate (FPR) of approximately 5%; SNPs with BF > 10 have a FPR of approximately 1%, and SNPs with BF > 1000 were considered to show association at a level of genome-wide significance.^{4, 30} In addition, associations between SCA severity and individual genes were tested using the SSE score, which uses a hypergeometric distribution to assess the probability that multiple SNPs within a single gene are all related to a phenotype.⁴

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Figure 1. Comparison of Plasma VCAM-1 and TNF-R1

Scatter-plot of log-transformed plasma levels of VCAM-1 and TNF-R1 among 24 subjects with SCA. Full circles represent subjects in the severe group (n=12), and empty boxes represent subjects in the mild group (n=12). Dotted lines mark the median log-transformed values for all 24 subjects.

TABLE 1
Summary of plasma biomarkers tested for potential association with SCA severity

All biomarker levels are expressed in [ng/mL], except NO, which is expressed in [μ M]. Biomarkers with significant associations with SCA severity are shaded in grey. All plasma markers were tested in all 24 subjects, except for ICAM-1 and P-Selectin, which were tested in 23 subjects.

Biomarker	Mild		Severe		P-Value
	Median	SD	Median	SD	
E-Selectin	81.60	± 37.46	79.73	± 77.29	8.9E-01
ICAM-1	320.40	± 122.33	354.55	± 150.80	2.7E-01
NO	15.76	± 11.15	11.37	± 9.43	4.1E-01
P-Selectin	39.67	± 40.79	39.32	± 18.83	3.2E-01
TNF- α	0.01	± 0.01	0.02	± 0.01	8.6E-01
TNF-R1	1.14	± 0.30	1.72	± 1.14	1.4E-03
VCAM-1	1157.25	± 486.54	2024.00	± 1366.35	1.8E-03

TABLE II
Genetic associations in biomarker and related genes

For each of the 16 genes identified as coding for or participating in the generation of the tested biomarkers, the number of SNPs tested in that gene is reported, along with the number found to be associated with SCA severity (*Sig. SNPs*, BF > 3 under any model of association). For each gene with at least one significant SNP, the SNP with the highest BF under any model of association with SCA severity is reported along with its BF

Gene	Number of SNPs	Number Significant	Top SNP	BF
Biomarker Genes				
<i>ICAM1</i>	3	0	NA	NA
<i>SELE</i>	3	0	NA	NA
<i>SELP</i>	17	0	NA	NA
<i>TNF</i>	1	0	NA	NA
<i>TNFRSF1A</i>	5	0	NA	NA
<i>VCAM1</i>	17	1	rs1041163	3.516
Associated Genes				
<i>ADAM17</i>	7	0	NA	NA
<i>ARF1</i>	3	0	NA	NA
<i>ARF3</i>	1	0	NA	NA
<i>ARFGEF2</i>	16	4	rs2273102	70.386
<i>ERAP-1</i>	9	0	NA	NA
<i>IL1B</i>	4	1	rs1143634	30.676
<i>NOS1</i>	38	2	rs527590	5.352
<i>NOS2A</i>	16	0	NA	NA
<i>NOS3</i>	2	0	NA	NA
<i>NUCB2</i>	7	0	NA	NA