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Fetal Hemoglobin Modulators May Be Associated With Symptomology of Football Players with Sickle Cell Trait

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Objectives: This study investigates whether genetic modifiers previously shown to influence adult fetal hemoglobin (HbF) levels and glucose-6-phosphate dehydrogenase deficiency were associated with variable symptomology in a small sample of collegiate football players with sickle cell trait.

Methods: Survey data on self-assessed symptoms and genotype data from five single nucleotide polymorphisms (SNPs) related to HbF production and two SNPs that cause glucose-6-phosphate dehydrogenase deficiency were collected from current and former college football players.

Results: In this sample, SNPs found within the β-globin gene cluster were found to be associated with a previous diagnosis of exertional sickling and experience of extreme heat during and after training. rs10189857 in the BCL11A gene was associated with body mass index and weight and with experiencing extreme thirst during and after training. No significant correlations were found between the other SNPs and symptoms within this sample.

Conclusions: These findings show that genetic variation known to affect sickle cell disease symptomology may partly explain why some football players with sickle cell trait experience adverse clinical outcomes during periods of extreme physical exertion and others do not.

Key Words: adult HbF production, athletes with SCT, developmental activation of hemoglobin genes, ECAST, sickle cell trait (SCT)

Sickle cell disease (SCD) results from the inheritance of two β-globin alleles (HbS) with an amino acid substitution at the sixth position, where valine replaces glutamic acid.[1] Clinicians have noted that patients with SCD differ in their disease expression,[2] mostly because of patients' levels of fetal hemoglobin

Key Points

- Exercise collapse associated with sickle cell trait (SCT) remains largely unexplained.
- This study assessed whether genetic modifiers previously shown to affect adult fetal hemoglobin levels and clinical variance among patients with sickle cell disease also are associated with variable symptomology in a small sample of collegiate football players with SCT.
- We genotyped collegiate football players with SCT for single nucleotide polymorphisms (SNPs) previously shown to affect levels of fetal hemoglobin and asked the athletes to complete a survey about the presence of symptoms associated with exercise collapse associated with SCT, and to compare themselves with their peers without SCT.
- We found statistically significant associations between SNPs and symptoms, and between one SNP and greater body weight and body mass index.
- We have demonstrated that SNPs previously shown to affect clinical variation in patients with sickle cell disease also are significantly associated with clinical variation in football players with SCT.

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(HbF), such that increasing levels are associated with better patient outcomes.[3,4]

Hemoglobin is one of the best-studied proteins in humans because of its role in distributing oxygen to the organs and other peripheral tissues and because of its clinical importance as the cause of inherited anemias, including SCD. Hemoglobin consists of four polypeptide chains, two α and two β chains ($\alpha_2\beta_2$). The α- and β-globin genes that produce these protein subunits are found in clusters on chromosomes 16 and 11, respectively. The expression of the three different α-like and five β-like globin genes involved in the production of hemoglobin is a classic example of cell and developmental-stage specific regulation.[5–7] In brief, during the embryonic stage of development, three types of embryonic hemoglobin (HbE) are produced through the activity of the HBZ (ζ), HBA, and HBA2 (α) α-like globin genes, and the HBE1 (ε), HBG1, and HBG2 (γ) β-like globin genes (as the tetramers $\zeta_2\varepsilon_2$, $\alpha_2\varepsilon_2$, and $\alpha_2\gamma_2$). During the first 3 months of gestation, production of HbE declines as HbF becomes predominant. HbF consists of two α and two γ chains ($\alpha_2\gamma_2$). In addition, during the fetal stage, the HBB (β) β-like globin gene is expressed at a low level and increases as birth approaches. After birth, a minor adult β-like gene, HBD (δ) also is expressed. During the first year of life, the adult forms of hemoglobin ($\alpha_2\beta_2$, $\alpha_2\delta_2$) become predominant. Most babies are born producing both fetal and adult hemoglobin; however, levels of adult hemoglobin 2 ($\alpha_2\delta_2$) remain low because of past changes in regulatory elements in the promoter of HBD.[8] In adulthood, most people produce >95% of adult hemoglobin 1 ($\alpha_2\beta_2$). Although HbF typically declines after birth, there is variation in the levels of HbF in adults. It has been shown that greater levels of HbF in adults ameliorate symptoms of hemoglobinopathies.[6,9–12]

Adults with HbF levels greater than ~5% have hereditary persistence of fetal hemoglobin (or HPFH) as a result of variation in the regulation of HBG.[13–15] Important modifiers of HbF production discovered thus far include the transcriptional repressors produced by the ZBTB7A and BCL11A genes and regulatory changes found in such regions as the HBS1L-MYB intergenic interval and in the promoter region of the HBG genes.[14,16–20] Researchers have quantified the variation in levels of HbF as a result of these single nucleotide polymorphisms (SNPs) to be up to 15% to 20%.[5,18] As such, although SCD is the result of a single Mendelian mutation, its severity and clinical manifestations are the result of polygenic interactions, including those that affect HbF, which contradict a simple Mendelian view.[21]

Although the diverse clinical manifestations of SCD have been established and explained for decades,[22] sickle cell trait (SCT)—the heterozygous condition—was previously believed to be uniformly benign. Most textbooks note that individuals with SCT are symptom free except under extreme conditions.[23–25] Such conditions include altitude-hypoxic environments[26,27] and periods of extreme exertion such as military[28–31] or sports training.[32] Following the work by Kark and colleagues during the 1980s, the US military engaged in an intervention study from 1982 to 1991 to determine whether better prevention of heat illness would reduce the deaths of recruits and reduce excess SCT deaths.[33] This study, which has not been published, showed that the intervention reduced or eliminated the excess mortality for recruits with SCT.[28] Because of this reduction in mortality, the US Army dropped SCT screening upon recruitment; however, as noted by Ferster and Eichner in 2012, exercise-related deaths continued.[28] A more recent study demonstrated that although US Army soldiers with SCT did not have higher mortality, they did have a higher risk of exertional rhabdomyolysis, which is the severe breakdown of skeletal muscle tissue precipitated by strenuous physical exertion.[34]

It is unfortunate that the increased risk of death associated with SCT in sports has not decreased.[35] The US Registry of Sudden Death in Athletes collected data for 31 years, 1980–2011. It notes that 23 deaths occurred in athletes with SCT, all of whom were African American and who died in remarkably similar circumstances involving noninstantaneous collapse with rapid deterioration following vigorous physical exertion at the early phase of training and conditioning. A majority of the athletes with SCT (19/23) were football players.[32] Notably, National Collegiate Athletic Association (NCAA) Division I football players with SCT had a risk of death that was 37 times higher than players who did not have SCT.[36] These alarming statistics led a 2012 expert panel of military and civilian experts in SCT and sports medicine to introduce the term "exercise collapse associated with SCT," or ECAST, to refer to the exercise-related complications experienced by the carriers of SCT.[37]

Most football players with SCT do not have clinical complaints or die; in fact, some have successful careers on professional teams. The question then becomes, what causes an individual with SCT to be at an increased risk of ECAST, whereas another exhibits no symptoms?[32] Most of the literature addresses this differential risk in terms of environmental factors such as hypoxia, extreme heat and humidity, dehydration, asthma, fatigue, lack of sleep, poor conditioning, and high exercise intensity.[37,38] To our knowledge, there has been no previous work testing the hypothesis that genetic modifiers of HbF production may at least in part explain why some but not all athletes with SCT experience exercise-induced complications.

The purpose of our study was to assess whether genetic modifiers previously shown to affect adult HbF levels and clinical variance among patients with SCD[9,17,39,40] also are associated with variable symptomology in a small sample of collegiate football players with SCT. We restricted our study to football players because of their increased risk of death[32,36] and to maintain homogeneity in our sample. We focused on symptoms associated with exercise-induced complications in football players with SCT. We hypothesized that a higher risk of experiencing at least some of these symptoms could be caused by the presence or absence of SNPs associated with HbF production.

Methods

The protocol for this study was reviewed and approved by the University of South Florida medical institutional review board

and reviewed at the Arizona State University Office of Research Integrity and Assurance on a yearly basis. The criteria for inclusion were being male, being at least 18 years of age, having had confirmation of SCT status with a blood test, and having played or currently playing American football. When the participants signed the consent form, they agreed to a statement to the effect that completion and submission of the survey and cheek swab constituted consent for the information to be used in a research paper in which their anonymity was ensured.

Study Design

We worked with current football players diagnosed as having SCT from blood tests and whose symptoms were self-assessed. The clinicians who designed and reviewed the survey were blinded to the genetic data of the participants, the geneticists were blinded to the clinical data of the participants, and the statistical analysts were blinded to both.

Study Sample

We embarked on an intensive recruitment campaign of NCAA Division I and II football teams from 2012 through 2016. Throughout 4 years of recruitment, the only successful strategy for obtaining participants was via university coaching and medical staff, who proved invaluable in encouraging current and even former team players with SCT to participate in our study. We decided to close recruitment when we obtained 31 samples from male football players (current and former), and we then proceeded with the data analysis. Because of missing data, our final sample size was 29.

Data Collection

Participants received a brief written survey, which asked them to check whether they experienced the presence or a higher frequency of symptoms than did their non-SCT teammates. These symptoms included drenching sweats, extreme thirst during and after training, hard muscles, whole-body muscle cramps, hematuria, and low-back pain. All of the symptoms were therefore self-assessed. The only symptom that was not self-assessed (although it was self-reported) was "Have you ever been told you suffered from exertional sickling?" The survey can be found in the Supplemental Digital Content (http://links.lww.com/SMJ/A148).

Because the most important modifiers of HbF production are the intergenic region HBS1L-MYB, the BCL11A gene, and the promoter region of the γ gene itself, we genotyped five SNPs found in these three regions. There are two SNPs at the promoter region of the γ gene within the β-globin gene cluster (rs7482144 and rs10128556), two at the BCL11A region (rs10189857 and rs4671393), and one at the HBS1L-MYB intergenic region (rs9402686).[18,41,42] To refer to the two forms of the SNP as ancestral or derived, we follow the assignment given by the SNP Database (https://www.ncbi.nlm.nih.gov/snp). The SNP Database is an active archive maintained by the National Center of Biotechnology Information, which determines which nucleotide has been changed (derived) from its original (ancestral) form. Also included in the study were two types of glucose-6-phosphate-dehydrogenase deficiency (G6PDD) because of the possibility of oxidative stress in red blood cells adding to the risk of exertional sickling.

DNA was extracted from participants' cheek swabs following the standard phenol-chloroform extraction protocol, and each sample was genotyped at the seven SNPs noted above using polymerase chain reaction with restriction digest and quantitative polymerase chain reaction-based genotyping methods. The five HbF SNPs are autosomal, so homozygous or heterozygous results were expected and observed in this sample. Conversely, the G6PD gene is found on the X chromosome; thus, hemizygous results (which should look the same as homozygosity) were expected in this all-male cohort. One individual, however, exhibited heterozygosity for both of the G6PDD SNPs, so standard methods were used to rule out possible contamination of the sample by female laboratory researchers. Although this anomaly was likely not due to contamination during DNA extraction and genotyping, this individual was nevertheless removed from the analyses of G6PDD to maintain homogeneity in the sample. A detailed description of the DNA extraction and genotyping methods used can be found in the Supplemental Digital Content (http://links.lww.com/SMJ/A148).

Analytic Methods

We determined whether the frequency of the symptoms from the survey was associated with the frequency of any of the SNPs using the Fisher exact test. We used SAS version 9.4 (SAS Institute, Cary, NC) for our statistical analyses.[43]

Results

Gene Frequencies

Table 1 shows the allele frequencies of five SNPs associated with HbF production and two SNPs associated with G6PDD for the entire sample (n = 29 for the HbF SNPs and n = 28 for the G6PD deficiency SNPs, because one male participant appeared to be heterozygous at this X-linked gene and was therefore excluded from the analysis). The frequencies of both β-globin gene cluster SNPs are identical as a result of their tight linkage; in other words, both SNPs are so close to each other in the gene that their frequency in this sample is identical.

All of the SNPs, except rs10189857 in the BCL11A gene, were in Hardy-Weinberg equilibrium, which means that the observed and expected frequencies are not significantly different. For rs10189857, however, the number of heterozygotes (AG = 16) exceeds that of the ancestral-type homozygotes (AA = 13), whereas no derived GG homozygotes are observed ($P < 0.05$). Previous studies have shown that the derived allele of the rs10189857 in the BCL11A SNP increases HbF in adults.

Table 1. Allele frequencies for the entire sample

SNP	rs7482144	rs10128556	rs10189857	rs4671393	rs9402686	rs1050829	rs1050828
Location	β-Globin gene cluster	β-Globin gene cluster	BCL11A	BCL11A	HBS1L-MYB	G6PD[a]	G6PD[a]
Alleles	G = ancestral; A = derived allele	C = ancestral; T = derived allele	A = ancestral; G = derived allele	G = ancestral; A = derived allele	G = ancestral; A = derived allele	A = ancestral; G = derived allele	G = ancestral; A = derived allele
Allele frequencies (N = 29)	$P = 0.88$, q = 0.12; GG = 22, GA = 7	$P = 0.88$, q = 0.12; GG = 22, GA = 7	$P = 0.72$, q = 0.28; AA = 13, AG = 16	$P = 0.81$, q = 0.19; GG = 19, GA = 9, AA =1	$P = 0.91$, q = 0.09; GG = 23, GA = 6	$P = 0.75$, q = 0.25; A = 21, G = 7	$P = 0.82$, q = 0.18; G = 23, A = 5
Hardy-Weinberg equilibrium	$\chi^2 = 0.55$, ns	$\chi^2 = 0.55$, ns	$\chi^2 = 4.21$; $P < 0.05$	$\chi^2 = 0$, ns	$\chi^2 = 0.39$, ns		

ns, not statistically significant; SNP, single nucleotide polymorphism.

[a]*n = 28 (29–1, where the possible XXY participant was excluded).*

The Homozygotes and Heterozygotes for Rs10189857 SNP at the BCL11A Differ in their Mean Body Mass Index (BMI) and Weight

We hypothesized that because heterozygotes for the rs10189857 SNP at the BCL11A produce more HbF, athletes would be able to train more and achieve greater musculature. To test this hypothesis, we divided participants into genotypic groups (AA and AG) for the rs10189857 SNP at the BCL11A and computed their mean height, weight, and BMI. According to our expectations, the heterozygotes were 10 k heavier ($P = 0.04$) and almost 2 BMI units larger than the homozygotes ($P = 0.04$), whereas their heights were virtually identical. A one-way test is appropriate in this case because our hypothesis was that the heterozygotes would achieve greater weight and BMI but not an equal or lesser weight or BMI with a median two-sample test (Table 2).

Association of Alleles with Symptoms

Three symptoms were significantly associated with the SNPs analyzed in this study. In the following, we present the odds ratios (ORs) according to how the odds of each symptom change from one SNP state to the other:

- A previous diagnosis of exertional sickling was associated with both SNPs in the β-globin gene cluster, where the derived alleles are protective of a diagnosis of exertional sickling ($OR_{ancestral\text{-}derived}$ 0.68, 95% confidence interval [CI] 0.51–0.9071). In our sample, the two individuals who had been diagnosed with exertional sickling were homozygote for the ancestral allele. Being heterozygote for the derived allele, which has been shown to result in higher levels of HbF, is protective of a previous diagnosis of exertional sickling.
- Experiencing extreme thirst during and after training was significantly associated with the rs10189857 allele in BCL11A. Of the 16 heterozygotes for the SNP, 10 reported the symptom ($OR_{ancestral\text{-}derived}$ 9.17, 95% CI 1.5–56.3). Being heterozygote for the derived allele, which has been shown to result in higher HbF levels, is associated with significant odds of extreme thirst during and after training.
- Experiencing extreme heat during and after training was significantly associated with the derived allele of rs7482144 at the β-globin gene cluster. Of the 4 individuals who reported the symptom, 3 were heterozygotes for this SNP ($OR_{ancestral\text{-}derived}$ 15.75, 95% CI 1.28–192.46). Being heterozygote for the derived allele, which has been shown to result in higher HbF levels, is associated with a significant odds of extreme heat during and after training.

Table 2. Comparison of the anthropometrics of the homozygotes and heterozygotes of the rs10189857 SNP at the BCL11A

Variable	Homozygotes	Heterozygotes	*P* (one-way)
Weight, k	86.7	96.2	0.044
BMI	25.625	27.84	0.044
Height, m	1.84	1.84	ns
Sample size	13	16	

BMI, body mass index; ns, not statistically significant; SNP, single-nucleotide polymorphism.

Lack of Association Between G6PDD SNPs and Symptoms

No significant association was found between the two G6PDD SNPs and any of the self-reported symptoms.

Discussion

This study is the first of which we are aware in which HbF modulators have been examined in SCT football players. Previous studies in cohorts of individuals with SCD have shown that higher HbF levels may be associated with milder symptomology. This line of research has established the importance of SNPs at the intergenic region HBS1L-MYB, BCL11A, and the β-globin gene cluster in modulating the levels of HbF, and therefore the expression of SCD.[13,24,44,45] The exact clinical effects of increased HbF levels in individuals with SCT are unknown; however, in discussing the results of this study, we operate within the assumption that increased HbF levels in individuals with SCT[32] also are clinically advantageous. In our sample, being a derived heterozygote at both SNPs in the β-globin gene cluster was significantly protective of a diagnosis of exertional sickling.

In the same manner, the rs10189857 at the BCL11A also is an important modulator of HbF levels in thalassemic patients.[46] In our sample, we found that being heterozygote for this SNP is associated with significantly higher BMI and weight (an obvious advantage in football). This result helps us understand why our sample is in Hardy-Weinberg disequilibrium for this SNP: In a highly competitive environment for this particular sport, individuals with SCT with higher levels of HbF will have better physiological means to succeed.

The question of why the heterozygotes of the rs10189857 at the BCL11A SNP also report extreme thirst during and after training more frequently than do ancestral homozygotes is important. We propose the following to explain these results: (1) Heterozygotes have greater exertional capability/limits because of their comparatively greater access to oxygen, which may result in greater conditioning with corresponding relatively higher weight and BMI. (2) Heterozygotes require additional exertional levels because they need to displace additional body mass in comparison to a lighter-weight individual performing a training task of the same intensity. (3) Heterozygotes have comparatively greater heat production and require greater water consumption because of this relatively higher exertion. A similar mechanism probably explains our result that experiencing extreme heat during and after training was significantly associated with the derived allele of rs7482144 at the β-globin gene cluster. This derived allele also has been reported to increase the production of HbF. It is likely that individuals who carry this SNP push themselves more because of their higher levels of HbF, and as a result, they report higher levels of extreme heat. These results suggest that these specific SNPs may be associated with these symptoms as a by-product of the other phenotypic manifestations of the SNPs.

All of the participants were sampled from NCAA Division I and Division II teams; therefore, these college athletes underwent extremely high levels of physical endurance. It is difficult to imagine that the homozygotes for the rs10189857 at the BCL11A SNP were randomly sampled from colleges that favor a small body size, whereas the heterozygotes for the rs10189857 at the BCL11A SNP were sampled from colleges that favor a large body size.

It is important to note that we applaud and support the efforts of medical staff who insist that players be hydrated and train slowly at the start of the season. We are complementing their work by pointing out that genetic variation also can play a role in the clinical manifestation of SCT during extreme physical exercise.

A potential weakness in our study is that we relied on the subjective reporting of symptoms and diagnosis by subjects. Our study also suffers from the shortcoming of having a small sample size (N = 29). Our small sample size stems from the tremendous difficulty in enrolling participants. After 4 years of aggressive recruitment campaign, we decided that it was time to publish our data, because a sample size of 30 is considered the point at which samples follow a normal distribution.[47] Moreover, as has been argued by others,[48] the usual expectation that every single paper conform to high sample sizes is unwarranted and detrimental to the development of innovative research avenues.

This study is the first of its kind to show that genetic modifiers related to variable HbF levels in adults do in fact influence the clinical manifestation of SCT in athletes. This is one of many calls for the recognition of SCT having actual clinical manifestations for some individuals. Future research with larger sample sizes, including nonathletes and individuals from numerous demographic populations, is required to support our results. It is time that the medical sciences acknowledge the variation within the clinical manifestations of SCT.

Conclusions

We have demonstrated that SNPs previously shown to affect clinical variation in patients with SCD also are significantly associated with clinical variation in football players with SCT.

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We dedicate this paper to the memory of the athletes with sickle cell trait who have died.

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